Quality of evidence used for the management of antimicrobial resistance in Australian animals

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Abstract

Integral to the success of surveillance programs is the quality of the measurement systems used to collect data. However, the performance of the measurement systems used to evaluate antimicrobial resistance and antimicrobial use is poorly defined. This thesis, therefore, examines the quality of evidence arising from the phenotypic assays and questionnaires used in the surveillance of animals.

The performance of disc diffusion was evaluated to determine its fitness-of-purpose as a source of data for clinical decision-making and surveillance. Zone diameter and minimum inhibitory concentration values obtained from the first Australia-wide prevalence studies of clinical *Escherichia coli* and *Staphylococcus pseudintermedius* were used to estimate the accuracy of disc diffusion relative to broth microdilution. Conventional measures of test accuracy were described, including diagnostic sensitivity, specificity, and area-under-the-receiver-operating characteristic (ROC) analysis. For most antimicrobials evaluated, disc diffusion was accurate at predicting the resistance of clinical *E. coli* and *S. pseudintermedius* that could otherwise be determined by broth microdilution. The assay performed strongly for ciprofloxacin and ceftiofur, and less favourably for amoxicillin-clavulanic acid, cephalothin, and cefoxitin. For *S. pseudintermedius* and oxacillin, the accuracy of broth microdilution was moderately better than disc diffusion relative to *mecA* real-time PCR. The precision of disc diffusion was investigated in a test-retest study using a linear mixed-model to estimate intra- and inter-laboratory agreement. Agreement was measured as repeatability (r) and reproducibility (R). The precision of disc diffusion was generally satisfactory for most antimicrobial agents, including ceftiofur (r=4.9mm, R=5.8mm) and gentamicin (r=4.9mm, R=5.4mm). However, the extent of variation in ampicillin (r=4.6mm, R=6.5mm) and trimethoprim-sulfamethoxazole (r=6.6mm, R=7.2mm) was of some concern.

The management of antimicrobial resistance is aided by the collection of data on the use of antimicrobial agents via questionnaires or other survey tools. In this thesis, the Australian beef feedlot sector was used as a case study to examine a common survey method in which multi-stakeholder engagement is expected, often leading to methodological constraints in survey design. Here, a mailed questionnaire was used to obtain information on antimicrobial use in beef feedlots. The response rate was 16.1%. For those responding to the survey, the use of antimicrobials was found to be appropriate for the purpose indicated, and there was a strong preference for drugs of low importance to human health. While the low response rate dictates that inferences could only be weakly extended to the broader beef feedlot population, the data was of value in informing the development of antimicrobial stewardship guidelines and acted as a staging position for further research into antimicrobial use in other animal sectors. However, more reliable methods of survey delivery should be considered for the on-going collection of antimicrobial use data at the farm-level.

Overall, this thesis concludes that for *E. coli* and *S. pseudintermedius*, susceptibility data from disc diffusion or broth microdilution generated in veterinary laboratories can contribute to national surveillance programs. This information, coupled with data from surveys of antimicrobial use at the farm-level, will be of substantial benefit to efforts aimed at managing antimicrobial resistance in animals.

Acknowledgments

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~ To Peter, Oliver, and Beatrix with love ~

"Begin at the beginning," the King said, gravely, "and go on till you come to an end, then stop". Lewis Carroll, Alice in Wonderland

List of Publications

Published Journal Articles

1. **Badger, S.**, Abraham, S., Saputra, S., Trott, D.J., Turnidge, J., Mitchell, T., Caraguel, C.G.B., Jordan, D., 2018. Relative performance of antimicrobial susceptibility assays on clinical *Escherichia coli* isolates from animals. *Veterinary Microbiology* 214, 56-64
2. **Badger, S.**, Abraham, S., O'Dea, M., Saputra, S., Abraham, R.J., Worthing, K.A., Norris, J.A., Trott, D.J., Jordan, D., Caraguel, C.G.B., 2019. Diagnostic accuracy of phenotypic assays for determining antimicrobial resistance status in *Staphylococcus pseudintermedius* isolates from canine clinical cases. *Veterinary Microbiology*. 234,101-109
3. **Badger, S.**, Abraham, S., Stryhn, H., Trott, D.J., Jordan, D., Caraguel, C.G.B., Intra- and inter-laboratory agreement of the disc diffusion assay for assessing antimicrobial susceptibility of porcine *Escherichia coli. Preventative Veterinary Medicine*. Volume 172, 15 November 2019, 104782 https://doi.org/10.1016/j.prevetmed.2019.104782
4. **Badger, S.**, Sullivan, K.F., Jordan, D., Caraguel, C.G.B., Page, S.W., Cusack, P.M.V., Firth, D., Trott, D.J. Survey of antimicrobial use in the Australian beef feedlot industry. *Australian Veterinary Journal*. Online version published 12 November 2019, https://doi.org/10.1111/avj.12889

Conference Proceedings

1. **Badger, S.**, Abraham, S., Saputra, S., Trott, D.J., Turnidge, J., Mitchell, T., Caraguel, C.G.B., Jordan, D., 2018. Performance of antimicrobial susceptibility assays on pathogenic *Escherichia coli* isolates from animals. Poster Presentation. Australian Centre for Antimicrobial Resistance Ecology (ACARE) Symposium. 24 June 2016, Adelaide, Australia.

2. **Badger, S**., Abraham, S., Saputra, S., Trott, D.J., Turnidge, J., Mitchell, T., Caraguel, C.G.B., Jordan, D., 2018. Performance of antimicrobial susceptibility assays on clinical *Escherichia coli* isolates from animals. Poster Presentation. 4th International Conference on the Responsible Use of Antimicrobial agents in Animals. 26-28 September 2016, The Hague, Netherlands.
3. **Badger, S**., Abraham, S., Saputra, S., Trott, D.J., Turnidge, J., Mitchell, T., Caraguel, C.G.B., Jordan, D., 2018. ROC the boat: antimicrobial susceptibility test performance. Oral Presentation. Australia and New Zealand College of Veterinary Scientists 2017 College Science Week Scientific Meeting, 6-8 July 2017. Gold Coast, Australia.
4. **Badger, S**., Abraham, S., Saputra, S., Trott, D.J., Turnidge, J., Mitchell, T., Caraguel, C.G.B., Jordan, D., 2018. Efficiencies in the surveillance of antimicrobial resistance. Oral Presentation. Australian Centre for Antimicrobial Resistance Ecology (ACARE) Workshop. 13-14 July 2017. Tanunda, Australia.
5. **Badger, S**. Antimicrobial Stewardship: Science and Regulation. Discussion Panel. Australian Veterinary Association National Conference, 7 May 2019. Perth, Australia

Local presentations

1. 'Surveillance of AMR in Australian Animals'. Department of Agriculture and Food, Western Australia workshop, 3 November 2016. Mandurah, Australia.
2. 'Integrated surveillance of AMR'. Department of Primary Industries and Regional Development workshop. 13 February 2019. Perth, Australia.
3. 'Antimicrobial resistance and antimicrobial agent use in livestock'. Department of Primary Industries and Regional Development workshop. 24 May 2019. Perth, Australia

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Skye Badger

Date: 15/07/2019

Thesis Context

The research areas presented in this thesis contributed to a national multi-institution collaboration which examined the phenotypic and genetic diversity of bacterial pathogens of importance to animals and evaluated the measurement systems used to generate data for surveillance programs. Participating organisations included The University of Adelaide, The University of Sydney, Murdoch University, the Australian Government Department of Agriculture and Water Resources, and the New South Wales Department of Primary Industries. From January 2013 to January 2014, veterinary diagnostic laboratories (n=22) in Australia contributed *Escherichia coli* and coagulase-positive staphylococci bacterial isolates from clinical cases to The University of Adelaide reference laboratory. Isolates underwent phenotypic, genotypic, and molecular testing at multiple institutions.

The quality of information derived from surveillance activities is dependent on the validity and reliability of the measurement tools used to collect information. High-quality data is necessary to implement strategies that manage antimicrobial resistance in animal populations. Hence, the research areas in this thesis focussed on three questions regarding the quality of the measurement systems used to generate data for surveillance programs:

1. Are antimicrobial susceptibility data generated from the disc diffusion assay sufficiently accurate for inclusion in national surveillance programs for animals? (Chapters 2 and 3)
2. How precise is disc diffusion when used in veterinary laboratories? (Chapter 4).
3. How well do stakeholder-driven questionnaires perform when used to collect farm-level antimicrobial use data? (Chapter 5).

A review of national surveillance programs and previous research into antimicrobial resistance and antimicrobial use in animals, along with a discussion on gaps in our collective knowledge is presented in Chapter 1. Chapters 2-5 present the objectives, methods, results, and discussion for each research area, while a detailed discussion, further research directions, and conclusions from this thesis are found in Chapter 6.

Chapter 1: Literature Review

Introduction

Antimicrobial resistance is a phenomenon that reveals the fragile interdependence between people, animals, and the environment; where overuse and misuse of these compounds have led to the rapid evolution and dissemination of antimicrobial resistance in bacterial populations (United Nations 2016; WHO 2015). Challenges associated with containing antimicrobial resistance are multidimensional. Of greatest concern are highly evolved mechanisms for the dispersal of resistance elements and genetic diversity in factors which rapidly select for resistance (Laxminarayan et al. 2013). Varying levels of awareness of antimicrobial resistance among medical professionals (Fletcher-Lartey et al. 2016; Labricciosa et al. 2018), veterinarians (Hardefeldt et al. 2017; Smith et al. 2018), and the community is a major barrier to changing behaviours and reducing use. Furthermore, deficiencies in data obtained by national surveillance programs impede our ability to mount an effective response to antimicrobial resistance and the over-consumption of antimicrobial agents. Consequently, there is a strong international consensus that integrated surveillance of people, animals, and the environment is essential if we are to fully comprehend the challenges associated with antimicrobial resistance (O'Neil 2016; OIE 2015b; WHO 2015).

This literature review introduces the central theme of this thesis – the quality of evidence arising from the measurement systems used to understand and manage antimicrobial resistance. Specifically, the diagnostic tests used to measure bacterial resistance, and the survey methods used to collect antimicrobial usage data at the farm-level. This literature review provides an overview of the surveillance of resistance in bacterial populations derived from animals; the antimicrobial susceptibility tests used to determine resistance; the methods for evaluating diagnostic test validity and precision; and the collection of antimicrobial usage data for inclusion in national surveillance.

Surveillance for antimicrobial resistance in the microbiota of animals

Epidemiologists are careful to distinguish between the terms 'surveillance' and 'monitoring'; however, in broader scientific usage, these terms are often synonymous despite differences in well-recognised definitions. Where surveillance is recognised as the systematic, on-going collection, analysis, interpretation, and dissemination of data to inform decision-making, stimulate action, and evaluate risk mitigation activities (Hoinville et al. 2013; Thacker, Qualters & Lee 2012; WHO 2015); monitoring occurs without a pre-defined risk mitigation plan or defined threshold level for intervention (Hoinville et al. 2013; Salman 2003). Surveillance can be categorised into five different purposes: (i) demonstration of freedom from disease, (ii) early detection of disease, (iii) prevalence of disease in a population, (iv) monitoring change in disease in a population over time, and (v) detection of cases to control disease (Dohoo, Martin & Stryhn 2009; Hoinville 2011). For antimicrobial resistance, the role of surveillance is to enhance our understanding of the epidemiology and risk factors which influence emergence and spread, and with this information, implement, and evaluate interventions which reduce the burden of resistance (WHO 2013).

Table 1 demonstrates a range of antimicrobial resistance surveillance activities which may fit within the five purposes of surveillance. From this table, comparisons can be made between each of the surveillance purposes (columns) and the epidemiological considerations (rows) required to design an effective surveillance program. For instance, the epidemiologic considerations necessary for the design of a surveillance activity to detect emerging resistance in a bacterial population are different from the design considerations for an activity which measures trends in resistance over time. In the former scenario, resistance is unknown or absent from a population, and the sampling strategy and sample size will be markedly different from the latter scenario where resistance is already well-characterised. It is clear from Table 1 that most national surveillance programs are in essence monitoring programs which have evolved to adopt elements of surveillance over time (McEwen, S, Aarestrup & Jordan 2006).

Table 1. Surveillance and epidemiologic characteristics of animal-focused antimicrobial resistance surveillance related to the five purposes of surveillance programs adapted from the WHO Guidance on the Integrated Surveillance of Antimicrobial Resistance in Foodborne Bacteria: Application of One-Health Approach (2017b) and the Animal Health Surveillance Terminology Report from the International Conference on Animal Health Surveillance (2011).

	Surveillance Purpose				
	Freedom from resistance	**Early detection of resistance**	**AMR prevalence/ distribution**	**Monitoring change**	**Case detection**
Surveillance characteristics:					
Political context	Management of outbreak, trade, control, prioritisation	Management of outbreak, trade, control, prioritisation	Prioritisation, control	Prioritisation, control, trade	Control, prioritisation, trade
Policy purpose	Public health information, risk analysis, design interventions, national database, measure success of interventions	Public health information, risk analyses, design interventions, guidance for prescribers, national database	Public health information, risk analyses, design interventions, guidance for prescribers, national database	Compare prevalence over time, public health information, risk analyses, design interventions, guidance for prescribers, national database	Determine interventions and measure success of interventions, risk analyses, national database
Surveillance objective	Demonstrate that a host population is free of a specified resistance gene for certain bacterial/host species	Detect emerging resistance in a bacterial species to trigger actions	Estimate prevalence and spatial distribution of resistance in a bacterial species/ host population at a point in time	Analyse changes in prevalence/ incidence of AMR in bacterial species/ host population over time	Find cases of AMR in bacterial species from a host population to intervene
Expected outcome of surveillance activity (and trigger level(s))	Probability of freedom of specified AMR in bacteria from host population	Identify new resistance genes (may move to case detection if want to contain/ prevent transfer of resistance)	Establish the prevalence of AMR in bacteria from host population.	Establish trends in the prevalence of AMR in bacteria from host population.	Identification of units of interest within host population (e.g., farm or individual animals) to contain spread
Anticipated actions taken	Based on political outcomes and risk to human health. Restrictions on certain antimicrobial classes in host population	Based on political outcomes and risk to human health. Restrictions on certain antimicrobial classes in host population	Restrictions on certain antimicrobial classes in host population. Methods to prevent food contamination	Restrictions on certain antimicrobial classes in host population. Methods to prevent food contamination	Restrictions on certain antimicrobials in host population, restrictions on trade of animals, methods to prevent contamination

	Surveillance Purpose				
	Freedom from resistance	**Early detection of resistance**	**AMR prevalence/ distribution**	**Monitoring change**	**Case detection**
Epidemiological characteristics:					
Context of surveillance purpose	Record free status from specified AMR genes	Detect novel or emerging AMR genes	Obtain AMR point prevalence data – phenotypic and/or genotypic data	Monitor trends in AMR in a population over time	Detect specified AMR genes to implement containment measures
AMR status	Absent	Absent	Present	Present	Present
Scope of surveillance activity	Ad hoc or continuous. Could be part of a portfolio of surveillance activities looking at one or more hazards. May be a single surveillance activity	Ad hoc or continuous. Could be part of a portfolio of surveillance activities looking at one or more hazards. May be a single surveillance activity	Ad hoc or one-off. Could be part of a portfolio of surveillance activities looking at one or more hazards	Continuous. Part of a portfolio of surveillance activities looking at one or more hazards	Continuous. Part of a network of surveillance activities to control hazard
Units of interest	Bacterial isolate Resistance gene Animal Herd Spatial region	Bacterial isolate Resistance gene Animal Herd Spatial region	Bacterial isolate Resistance gene Animal Herd Spatial region	Bacterial isolate Resistance gene Animal Herd Spatial region	Bacterial isolate Resistance gene Animal Herd Spatial region
Host population stream	Diseased – clinical Healthy – farm, abattoir	Diseased – clinical	Healthy – farm, abattoir, retail	Healthy – farm, abattoir, retail	Healthy – farm Diseased – clinical
Sampling strategy (for selection of study population)	Probabilistic Risk-based Multi-stage	Probabilistic Risk-based Multi-stage Non-probabilistic Convenience	Probabilistic Representative Multi-stage	Probabilistic Representative Multi-stage	Non-probabilistic Purposive/ targeted
Sample size coverage required to meet objective	Medium (for rare occurrence)	Medium-high (for rare occurrence)	Low-medium (for higher prevalence)	Low-medium (for higher prevalence)	Medium-high (for control/ eradication)
Origin of data	Active Passive (lab data)	Active Passive (lab data)	Active	Active Passive (lab data)	Active
Sampling method	Bacterium or pooled sampling	Bacterium or pooled sampling	Bacterium or pooled sampling	Bacterium or pooled sampling	Bacterium or pooled sampling

	Surveillance Purpose				
	Freedom from resistance	**Early detection of resistance**	**AMR prevalence/ distribution**	**Monitoring change**	**Case detection**
Case definition (microbiological interpretive criteria)	Lab test confirmed: Phenotypic Genotypic (PCR, WGS), ECOFF	Lab test confirmed: Phenotypic Genotypic (PCR, WGS), ECOFF	Lab test confirmed: Phenotypic ECOFF	Lab test confirmed: Phenotypic ECOFF	Lab test confirmed: Phenotypic Genotypic (PCR, WGS), ECOFF
Data measurements	Presence or absence	Presence or absence	Prevalence, distribution	Prevalence, Incidence	Prevalence count
Examples of surveillance activities	There are no proof of freedom activities. However, proving freedom from carbapenem-resistant Enterobacteriaceae (CRE), colistin (*mcr-1*) and vancomycin-resistant (VRE) *E. coli* from livestock will be desirable	Fluoroquinolone resistance in food animals; ESBLs in salmonella from food animals in Australia; National Alert System for Critical Antimicrobial Resistances (CARAlert) (2019)	Prevalence of AMR in *Salmonella* and *E. coli* from cattle in Australia in 2015 (Barlow et al. 2015); poultry (Barton & Wilkins 2001) (Australian Chicken Meat Federation 2018); pigs (Kidsley et al. 2018)	National surveillance reporting prevalence of AMR in *E. coli* from pigs in Denmark since 1995 (DANMAP 2017); Canada since 1997 (Government of Canada 2017)	Methicillin-resistant *Staphylococcus aureus* in animals (Jordan, D. et al. 2011; Sahibzada et al. 2017); National program to control ceftiofur-resistant *Salmonella* enterica serovar Heidelberg in poultry in Canada (Dutil et al. 2010)

AMR, antimicrobial resistance; ECOFF, epidemiological cut-off value; PCR, polymerase-chain-reaction; WGS, whole-genome sequencing

Australian Chicken Meat Federation, 2018. Surveillance for antimicrobial resistance in enteric commensals and pathogens in Australian meat chickens. October 2018. ACMF. https://www.chicken.org.au/wp-content/uploads/2018/10/Chicken-Meat-AMR-survey-Final-report.pdf Accessed 12/04/2019.

Australian Commission on Safety and Quality in Health Care, 2019. CARAlert update 10: 1 November 2018–31 December 2018. Sydney: ACSQHC; 2019

Barlow, R.S., McMillan, K.E., Duffy, L.L., Fegan, N., Jordan, D., Mellor, G.E., 2015. Prevalence and Antimicrobial Resistance of Salmonella and Escherichia coli from Australian Cattle Populations at Slaughter. Journal of Food Protection 78, 912-920.

Barton, M., Wilkins, J., 2001. Antimicrobial agent resistance in bacteria isolated from poultry: A report for the Rural Industries Research and Development Corporation.

DANMAP, 2017. DANMAP 2016 - Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. ISSN 1600-2032.

Dutil, L., Irwin, R., Finley, R., Ng, L.K., Avery, B., Boerlin, P., Bourgault, A.M., Cole, L., Daignault, D., Desruisseau, A., Demczuk, W., Hoang, L., Horsman, G.B., Ismail, J., Jamieson, F., Maki, A., Pacagnella, A., Pillai, D.R., 2010. Ceftiofur resistance in Salmonella enterica serovar Heidelberg from chicken meat and humans, Canada. Emerg Infect Dis 16, 48-54.

Government of Canada, 2017. Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) 2015 Annual Report. ISSN:1925-9859. In: Public Health Agency of Canada (Ed.), Guelph, Ontario.

ICAHS, 2013. Animal Health Surveillance Terminology: Final report from Pre-ICAS Workshop (Version 1.2). In: Hoinville, L. (Ed.), International Conference on Animal Health Surveillance.

Jordan, D., Simon, J., Fury, S., Moss, S., Giffard, P., Maiwald, M., Southwell, P., Barton, M.D., Axon, J.E., Morris, S.G., Trott, D.J., 2011. Carriage of methicillin-resistant Staphylococcus aureus by veterinarians in Australia. Australian veterinary journal 89, 152-159.

Kidsley, A.K., Abraham, S., Bell, J.M., O'Dea, M., Laird, T.J., Jordan, D., Mitchell, P., McDevitt, C.A., Trott, D.J., 2018. Antimicrobial Susceptibility of Escherichia coli and Salmonella spp. Isolates From Healthy Pigs in Australia: Results of a Pilot National Survey. Frontiers in Microbiology 9.

Sahibzada, S., Abraham, S., Coombs, G.W., Pang, S., Hernandez-Jover, M., Jordan, D., Heller, J., 2017. Transmission of highly virulent community-associated MRSA ST93 and livestock-associated MRSA ST398 between humans and pigs in Australia. Scientific reports 7.

Over 20 countries have national antimicrobial surveillance programs which collect data on bacteria from people, animals, and food products. In most countries, national surveillance is focussed on pathogenic bacteria from people and zoonotic and commensal bacteria from healthy food animals and retail meat products. Bacteria from food animals and retail meat products of most interest are *Salmonella* spp., *Campylobacter* spp., *Escherichia coli,* and *Enterococcus* spp. These bacteria are included as they are thought to play a role in the transfer of genetic resistance elements to humans (OIE 2015b; WHO 2001). Countries without national surveillance programs, Australia included, have conducted small studies of limited time coverage to assess antimicrobial resistance in bacteria from food animals and retail meat products (Australian Chicken Meat Federation 2018; Barton & Wilkins 2001; Jordan, D 2003). Noticeably absent from most national surveillance programs is information on pathogenic bacteria from animals, with just a few countries, such as Denmark (DANMAP 2017), Germany (GERM-VET 2018), Norway (NORM/NORM-VET 2017), Sweden (Swedres-Svarm 2017), and France (RESAPTH 2017), monitoring some pathogenic bacteria from animals. The lack of data about pathogens represents a major knowledge gap regarding the prevalence of antimicrobial resistance in animals. A concerted effort is needed to obtain data on antimicrobial resistance in animal pathogens if we are to advance our understanding of the health risks posed to animals, people, and the environment (Barber, Miller & McNamara 2003; European Union 2013; Guardabassi, Schwarz & Lloyd 2004).

A lack of standardisation in national surveillance programs is a significant challenge for coordinated action on antimicrobial resistance (Bax et al. 2001; Fluit et al. 2006; Shaban et al. 2014; White et al. 2001; WHO 2017a). The absence of standardisation, particularly in sampling procedures and laboratory testing methodologies, is a barrier to data-sharing and comparability of resistance levels between countries (WHO 2013). At present, the only comparable antimicrobial resistance surveillance data are from programs conducted in EU-member states, the United States, and Canada (WHO 2013). The OIE (2016) and WHO (2017b) have published guidelines on the standardisation of antimicrobial surveillance, and the European Parliament

has enacted legislation to impose standardisation of antimicrobial resistance surveillance in all member states (European Union 2013). However, countries may have different objectives for surveillance, access to funding, legislation, infrastructure, and farm practices which will affect the design, and standardisation, of surveillance activities and therefore the ability to compare surveillance data.

Surveillance objectives should be specific, measurable, and time-dependent and form the basis for planning and evaluation. The broadly agreed objectives for most surveillance programs of antimicrobial resistance include the (i) determination of resistance in a population, (ii) monitoring changes in resistance, (iii) detection of new mechanisms of resistance, (iv) investigation of the evolution of resistance, (v) determination of antimicrobial use patterns, and (vi) development and monitoring of interventions, (Franklin et al. 2001; OIE 2015b; Silley, Simjee & Schwarz 2012; WHO 2017b). For the most part, the objectives listed above are comparable to the purposes of surveillance outlined in Table 1, specifically the early detection of resistance, prevalence of resistance in populations, and monitoring change over time. However, for many national surveillance programs, the program objectives are often criticised for lacking clarity and relevance for animal populations (Jordan, D 2003; Lewis 2002). Poorly defined objectives in surveillance programs can lead to weak study design and impact on the quality and comparability of data outputs (Fluit et al. 2006; Franklin et al. 2001).

Central to surveillance is the collection of objective and robust data, which can be achieved by a well-designed sampling strategy and the use of accurate and reliable measurement systems. Here, sampling is addressed. The sampling strategy should have two essential features: population representativeness and adequate sample size. Representativeness ensures the sample subset is, as much as possible, an accurate and unbiased reflection of the population from which the sample group is drawn; while a statistically appropriate sample size results in valid data outputs (Dohoo, Martin & Stryhn 2009; Franklin et al. 2001; WHO 2013). Flawed study design can result in weak data and inferior decision-making (Rempel, Pitout & Laupland 2011). Balancing the cost of sampling and the usefulness of the data is a considerable

challenge, especially in resource-challenged settings. Under-resourcing constrains the number of samples collected, sites visited (e.g., farms, abattoirs) and regularity of sample collection. Notwithstanding limitations in sampling, data outputs can still be useful, although they may be insufficient to address the objectives of a national surveillance program.

Where representativeness in sampling is unachievable, as may be the case for certain surveillance activities in animal populations, risk-based sampling can be an efficient and resource-saving approach. This is particularly so for demonstrating freedom from disease or absence of infection (e.g., the absence of certain multi-drug resistant genes in bacteria from a food animal species). Risk-based sampling has been described as an approach whereby the sampling strategy applied to different strata in a population is based on the probability of infection (or carriage of resistance genes) in that strata (Cameron, AR 2012). For example, cattle reared in an extensive grazing system will likely have a lower risk of acquiring and disseminating resistance genes compared to cattle kept in a feedlot where infectious diseases and exposure to antimicrobial agents may be high. Therefore, the sampling strategy in extensively grazed cattle and feedlot cattle will be different based on their perceived level of risk of exposure to antimicrobial agents, selection pressure in bacterial populations, and carriage of resistant genes.

Sampling design is often considered the most common source of systematic error (bias) in population measurements, yet it has historically received scant consideration when designing antimicrobial resistance surveillance activities (Dunlop et al. 1999; Jordan, D 2003). Inappropriate sampling occurs when it is incorrectly assumed that an event of interest (e.g., occurrence of resistant bacteria) is randomly distributed within the population of interest (Dohoo, Martin & Stryhn 2009). The non-random distribution of infectious agents is known as clustering. While it generally holds that the health status from animals within herds are more alike than the health status of animals from separate herds, several studies have also reported on clustering of resistant bacteria within faecal samples derived from individual animals and groups of animals from the same herd (Benedict et al. 2015; Dunlop et al. 1999; Humphry et

al. 2018). Thus, clustering can occur at multiple 'levels'. Most national antimicrobial resistance surveillance programs implicitly assume homogeneity in bacterial populations at the farm-level and define the epidemiological unit of interest as a single bacterial isolate per farm. However, this approach ignores the phenomenon of clustering and will reduce the likelihood of identifying low-level or emerging bacterial resistance (Dunlop et al. 1999; Humphry et al. 2018; Persoons et al. 2011; Vieira et al. 2008).

By assuming homogeneity at the farm-level, most national programs utilise standard sample size calculations to determine the number of bacteria required to evaluate resistance (Caprioli et al. 2000; Davison, Low & Woolhouse 2000; European Food Safety Authority 2012). This assumption underestimates the sample size required for any given level of accuracy when clustering is present, and with this approach, it may not be possible to obtain an accurate assessment of the prevalence of antimicrobial resistance in a bacterial population derived from an animal species (Jordan, D 2003; Persoons et al. 2011; Shaban et al. 2014). Estimates of the prevalence of antimicrobial resistance from food animals could be less biased if the sample size was increased or the sampling unit was based on a pooled sample at the farm-level where clustering could be accounted. Techniques described by Dunlop et al. (1999), Wagner et al. (2002), Benedict et al. (2013), and Humphry et al. (2018) demonstrate the suitability of pooled faecal sampling in estimating the low-level prevalence of antimicrobial resistance in bacteria from animals. Coupled with affordable and reliable high-throughput laboratory testing, pooled sampling at the farm-level could overcome current deficiencies in the reporting of prevalence of resistance in food animals. Examination of ways to increase the sample size by using pooled faecal sampling and high-throughput laboratory testing was an original objective of this thesis; however, at the time of completion of the research phase, suitable protocols for exploiting the robotic technology at the Murdoch University Antimicrobial Resistance and Infectious Diseases (AMRID) Research Laboratory were still under development.

Antimicrobial susceptibility data which routinely accumulates in veterinary diagnostic laboratories could be used to enhance the current surveillance effort in animals. The collection of clinical data from laboratories is a form of passive surveillance (Dohoo, Martin & Stryhn 2009; Thrusfield 2007). The strength of passive surveillance lies in its low cost and potential to identify emerging or rare resistance in bacterial pathogens, an objective of all existing national surveillance programs (Mather et al. 2016). However, passive surveillance is not without bias, since such data is typically derived from clinically unwell individuals, and there is a reliance on veterinarians (and consenting owners) to submit samples for investigation. For veterinary laboratories to be a reliable data source, a high level of confidence is needed in the performance of the phenotypic assays used in these laboratories. Specifically, the assays must be accurate and reliable within- and between-laboratories (Bax et al. 2001).

Antimicrobial susceptibility assays

In clinical settings, phenotypic antimicrobial susceptibility testing is used to determine the susceptibility of a bacterial isolate to an antimicrobial agent as an aid to therapeutic decision-making. While in surveillance, the use of phenotypic assays is different. Here, the assays are used to gather temporal and spatial data to aid the design of policies and interventions.

The two most common phenotypic assays used in veterinary laboratories are broth microdilution and disc diffusion. Broth microdilution is the reference standard to which all other phenotypic assays are compared (ISO 2006), and is preferred for national surveillance as it generates quantitative data based on the minimum inhibitory concentration (MIC) (OIE 2018b). However, disc diffusion, which measures the zone of inhibition around an antimicrobial agent-infused disc on agar, is commonly used in veterinary laboratories. This is because disc diffusion is affordable, customisable for a range of bacteria and antimicrobial agents, and requires minimal investment in equipment compared to broth microdilution. Indeed, a recent survey of American veterinary laboratories reported 71% of respondents performed disc diffusion

(Dargatz, Erdman & Harris 2017), while in Australia, all veterinary laboratories reported using disc diffusion to evaluate antimicrobial susceptibility (Hardefeldt et al. 2018).

Bacterial isolates are usually described as being “susceptible”, “intermediate”, or “resistant” to an antimicrobial agent when the interpretative criteria, known as clinical breakpoints, are applied. Clinical breakpoints are used to determine an isolate’s susceptibility to the antimicrobial agents tested and to select the most suitable therapeutic agent. The clinical interpretation of disc diffusion results is considered comparable to those from broth microdilution, providing international standards for performing the assay such as those published by the Clinical and Laboratory Standards Institute (CLSI) or EUCAST are observed (Lestari et al. 2008; Matuschek, Brown & Kahlmeter 2014; Turnidge & Paterson 2007). Clinical breakpoints are determined by expert committees which consider MIC distributions, pharmacokinetics/ pharmacodynamics of the antimicrobial agent, and clinical outcomes (Turnidge & Paterson 2007). However, there are few clinical breakpoints specific to veterinary isolates/ drug combinations. Over-reliance on human breakpoints has led to challenges with the clinical interpretation of veterinary bacterial pathogens. If human breakpoints are inappropriate for a veterinary bacteria/ antimicrobial combination, the test result will be of limited value to the clinician. Unsuitable breakpoints can lead to inappropriate selection of antimicrobial agents and potentially select for resistance (Toutain et al. 2017).

When setting clinical breakpoints for veterinary medicine, several issues need to be considered. Namely, antimicrobial agents may be administered to multiple animal species by various routes, dose rates, and using formulations with short-acting or long-acting durations of action. Also, bioavailability is variable depending on the species, breed, and animal behaviours (Toutain et al. 2017). Hence, the ongoing appraisal of the most appropriate veterinary-specific breakpoints is essential, particularly for clinical decision-making and the early detection of rare and emerging resistance.

The modified error-rate bounding method described by Brunden, Zurenko and Kapik (1992) is commonly used by international standards groups such as CLSI to help determine 'best-fit' zone diameter clinical breakpoints. 'Best-fit' breakpoints are based on predefined acceptable levels for misclassification errors (i.e., very major, major, minor errors) described by ISO (2006). Several superior model-based approaches have been developed to introduce robustness to clinical breakpoint determination (Craig 2000; DePalma, Turnidge & Craig 2017; Kronvall, Giske & Kahlmeter 2011).

Epidemiologic cut-off values (ECOFFs) are used as interpretative criteria in surveillance settings. The ECOFF separates bacteria into wild-type and non-wild type populations (Kahlmeter et al. 2003). Usually, bacteria assigned to the wild-type population do not harbour resistance genes or resistance-mediating mutations, while those of the non-wild type population commonly do. Since ECOFFs are not determined by the same criteria used to establish clinical breakpoints they are less useful for therapeutic decision-making. Clinical breakpoints and ECOFFS may be closely related for some antimicrobial agents and bacterial species, however for other combinations, both types of interpretative criteria are far apart. Direct comparison of susceptibility data is not always possible as studies use different clinical breakpoints (ECDC/EFSA/EMA 2015; Silley 2012), so ECOFFs are recommended for use in surveillance to enable direct comparison of bacterial/ antimicrobial resistance datasets (Davison, Low & Woolhouse 2000; OIE 2016; Schwarz et al. 2010; Silley, Simjee & Schwarz 2012). However, fewer ECOFFS are presently available for animal bacteria, making reporting and comparison of resistance levels between datasets challenging. For example, at the time of writing, there was no zone diameter ECOFF published for *E. coli* and ceftiofur, a third-generation cephalosporin used in food animals and categorised as highly important for human health by the Australian Strategic and Advisory Group on Antimicrobial Resistance (2018) and the OIE (2015a).

Many national surveillance programs utilise genotypic and molecular tests to identify acquired resistance genes in bacterial isolates from animals, such as the *mecA* gene associated with methicillin-resistance in staphylococci species (Swedres-Svarm 2017), and the gentamicin resistance gene *aph(2")* in *Campylobacter coli* isolated from retail chicken meat (USDA 2017). When used together, phenotypic and molecular testing offers the best information on the management of multi-resistant bacterial infections and detection of new mechanisms of bacterial resistance. However, veterinary laboratories have been slow to adopt the technology, with Dargatz, Erdman and Harris (2017) reporting 6% of respondents to a survey of America veterinary laboratories, used molecular technologies. In Australia, Hardefeldt et al. (2018) reported very few veterinary laboratories utilise such technologies. Difficulties in the adoption of molecular technologies will need to be overcome before veterinary laboratories can incorporate them into testing regimes. This includes understanding the relationship between phenotypic testing and resistance genes in different bacterial species, the development of user-friendly platforms for interpretation of the data, and the cost of infrastructure and labour to operate the equipment (Didelot et al. 2012; Frickmann, Masanta & Zautner 2014).

There is little consensus on standard antimicrobial panels to include for surveillance of animal-derived bacterial species. Also, most antimicrobial agents tested in national surveillance programs focus on classes of importance to human health. To address the lack of standardisation, the European Parliament passed legislation which requires member states to test a standard panel of 14 antimicrobial agents for animal-derived *Salmonella* and *E. coli,* and 12 antimicrobial agents for *Enterococcus* spp (European Union 2013). Some countries, such as Denmark, test more antimicrobial agents than specified in the legislation (DANMAP 2017). While decisions regarding which antimicrobial agents to test can be difficult, testing large numbers of antimicrobial agents is both unnecessary (many antimicrobials have similar *in vitro* activities) and cost-prohibitive (Silley, Simjee & Schwarz 2012). From an epidemiological viewpoint, one could argue the 'over-testing' of isolates is inefficient when attempting to manage a complex antimicrobial resistance surveillance program, particularly for countries

with limited resources and infrastructure. A global agreement to test a restricted number of antimicrobial agents per bacterial species and animal host will result in cost and time savings, enable comparative analysis of datasets, and data generated will be targeted and relevant to surveillance objectives.

Diagnostic test evaluation

An understanding of the performance of laboratory tests is critical to the design and interpretation of surveillance and monitoring activities. Uncertainty regarding the performance of diagnostic tests raises questions about the quality of data collected and reported by national surveillance programs. Accurate data on animal-derived bacteria is essential as it is used to inform antimicrobial use policies related to food animals, thus having ramifications for public health (Tang et al. 2017). Diagnostic test performance is described by its accuracy and precision. Accuracy refers to the deviation of a measurement from its 'true' value, while precision refers to the closeness of measurements from the same sample (ISO 1994; OIE 2018a). All diagnostic tests are subject to random and systematic errors, resulting in potential misclassification of test values (Gardner, I.A. & Greiner 2000). For phenotypic antimicrobial susceptibility tests, misclassification may result in a bacterial isolate being categorised as susceptible when it is truly resistant to an antimicrobial agent (worst case scenario in clinical settings) or vice versa. Measurement errors can be complex to define, especially for antimicrobial susceptibility tests where antimicrobial resistance is rapidly evolving, and the criteria used to evaluate resistance status is continually changing.

Accuracy

Accuracy is traditionally described by diagnostic sensitivity and specificity. Put simply, diagnostic sensitivity refers to the ability of a test to correctly identify subjects with the disease of interest (e.g., phenotypically resistant), while specificity relates to the ability of a test to correctly identify subjects free of disease (e.g., phenotypically susceptible). Estimation of

diagnostic sensitivity and specificity is conditional on two factors, (i) knowledge of the true status of a bacterium (determined by a reference test), and (ii) the threshold value (e.g., clinical breakpoint) used to dichotomise measurement values into test positive (resistant) and test negative (susceptible) groups (Dohoo, Martin & Stryhn 2009; Greiner & Gardner 2000). Ideally, the reference test is perfect, such that the classification is always correct. However, most reference tests are less than perfect and subject to systematic error (Gart & Buck 1966). When errors in the reference test are disregarded, bias is present in the accuracy estimates, and these estimates are at best 'relative'. This bias will be such that the accuracy of the comparator test can never exceed the errors inherent in the reference test (Enoe, Georgiadis & Johnson 2000; Greiner & Gardner 2000). In antimicrobial susceptibility testing, broth microdilution is considered the reference test against which all other assays are compared. However, the accuracy of broth microdilution is not well understood and probably imperfect since there are few tests considered superior other than a limited number of genetic or molecular tests.

The threshold value used to dichotomise test values has a critical influence on estimates of diagnostic sensitivity and specificity (Greiner & Gardner 2000). Depending on the distribution of measurement values in a sampled population, the positioning of the threshold value will result in varying levels of misclassification errors. Figure 1 shows three different zone diameter distributions, with the top graph demonstrating well-separated distributions. Few misclassification errors occur when a clinical breakpoint is located somewhere between the two populations. In the middle figure, where there is complete overlap, the measurement is of no benefit as it is unable to discriminate between isolates that are resistant or susceptible. In the bottom graph, a decision about the location of a clinical breakpoint will depend on which type of misclassification error is more tolerable. For example, in clinical settings, a breakpoint that results in a high number of false negative (i.e., false-susceptible) errors is unacceptable.

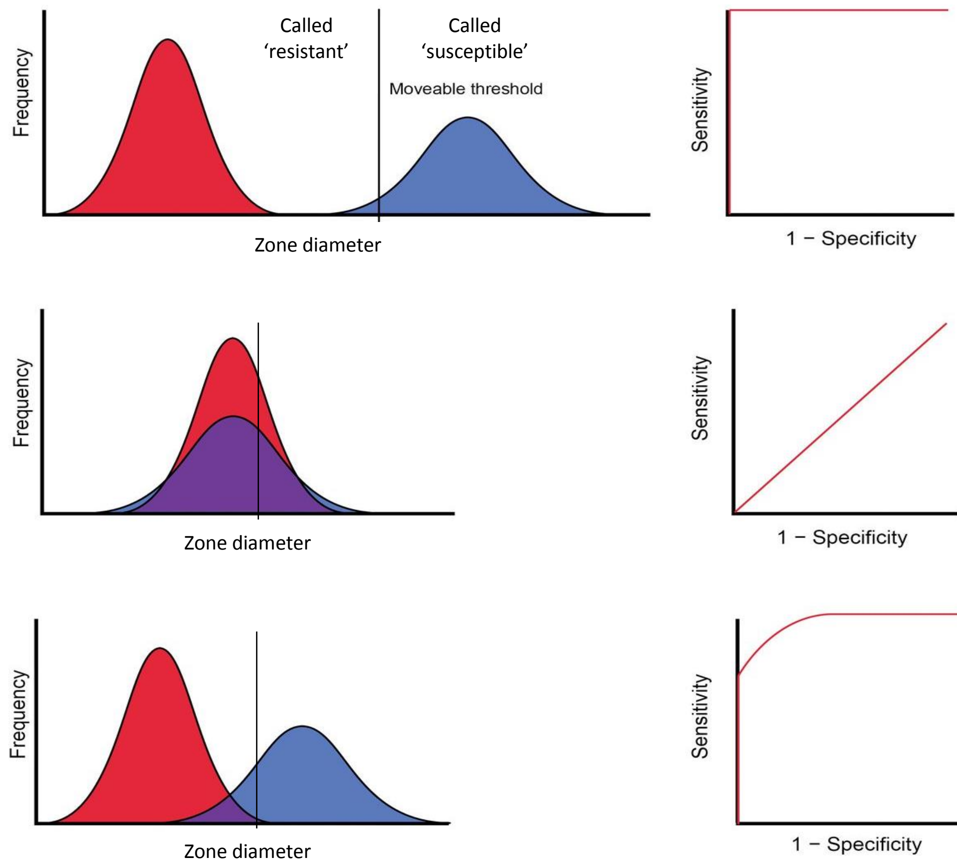

Figure 1: Hypothetical distributions of resistant (red) and susceptible (blue) bacterial isolates with corresponding receiver-operator characteristic (ROC) plots. Adapted from (Schwartz 2012).

The receiver-operating characteristic (ROC) analysis addresses issues associated with dichotomising continuous data since it is independent of the threshold-value (Greiner, Pfeiffer & Smith 2000). Accuracy is measured by the ROC area-under-the-curve (AUC), which describes a test's ability to separate a group into those with and without the disease (Gardner, I. A. & Greiner 2006). In ROC analysis, each point on the curve represents a sensitivity and specificity pair corresponding to the decision threshold. A test with perfect discrimination (AUC = 1) has a curve which touches the top left corner of the graph, while a test with no discriminatory power (AUC = 0.5) has no curve (Swets 1988). Figure 1 demonstrates the ROC curve expected with each of the three distributions. The AUC = 1 (100% diagnostic sensitivity and specificity) when there is no overlap between populations. However, the AUC estimate suffers depending on the extent the two groups overlap, with an AUC of 0.5 occurring when

the two populations completely overlap. Despite the advantages of using ROC analysis to determine test accuracy, it does not discriminate between misclassification errors (Greiner & Gardner 2000). Further, ROC analysis relies on the use of a 'perfect' reference test, which is not always available. Although ROC analysis is integral to sensitivity and specificity estimates for continuous outcome tests, it is not widely used in veterinary diagnostic test evaluation. This is undoubtedly the case for the evaluation of the antimicrobial susceptibility tests where measures of accuracy are infrequently reported, particularly for veterinary bacterial pathogens.

An advantage of reporting robust relative sensitivity and specificity estimates is that apparent prevalence (derived from the comparator test) can be corrected to true prevalence (derived from the reference test) (Rogan & Gladen 1978). Correcting apparent prevalence to true prevalence allows for direct comparison of prevalence estimates from two different tests (e.g., disc diffusion and broth microdilution). This feature is highly useful in surveillance where estimates of prevalence are important epidemiologic indicators of resistance in the population.

Precision

Fundamental to the assessment of precision is the statistical estimation of reliability or agreement by taking repeated measurements of the same subject (e.g., a bacterial isolate) within (repeatability) and between (reproducibility) multiple laboratories. Reliability describes the ratio of variability between subjects to the total variability of all measurements, while agreement quantifies the degree to which two measurements are identical (Kottner et al. 2011). Reliability studies estimate intra- and inter-laboratory reliability from intraclass correlation coefficients (ICC) (Barnhart, Haber & Lin 2007; Bartlett & Frost 2008). The reliability ICC describes the correlation between repeated measurements on the same sample and across multiple samples and takes on values between zero and one, with one (i.e., high reliability) representing no measurement error and zero indicating all variability is due to measurement error (de Vet et al. 2006).

In contrast, agreement studies are designed around the notions of repeatability and reproducibility which describe within- and between-observer variability (Barnhart, Haber & Lin 2007; ISO 1994). Agreement is a characteristic of the test and does not depend on the population in which measurements are made unless bias is present or the true value of the measurement varies (Bartlett & Frost 2008). Agreement is measured in the same units as the test. Repeatability studies have strict conditions on the measurement of precision (e.g., the same technician, same equipment, short times intervals), while reproducibility studies allow for changing conditions including different laboratories, and technicians (ISO 1994). Reproducibility studies are particularly useful when inferences are made on the wider population of potential observers such as veterinary laboratories operating in a national network (Barnhart, Haber & Lin 2007; Bartlett & Frost 2008).

There is no standard approach to the statistical exploration of reliability and agreement, as study objectives and design factors such as the sampling strategy and type of data collected have a large bearing on assumptions used in estimation model-building (Kottner et al. 2011). When reviewing literature on the precision of the disc diffusion assay, previous studies have tended to limit evaluation to well-characterised strains such as the ATCC quality control strains recommended by CLSI or EUCAST (Hombach et al. 2017; Hombach, Zbinden & Bottger 2013; Idelevich et al. 2016; Lehtopolku et al. 2012; Matuschek, Brown & Kahlmeter 2014; Medeiros & Crellin 2000; Murray, Zeitinger & Krogstad 1982).

Surveillance of antimicrobial use in animals

There are many motivations in administering antimicrobial agents to animals. Of most concern to the wider community is the use of antimicrobial agents considered medically important to people and the non-therapeutic use of antimicrobial agents for growth promotion (McEwen, SA & Collignon 2018; O'Neill 2014; WHO 2013). Much of this concern is centred on the poorly-defined contribution antimicrobial use in animals makes toward the development

of resistance in human bacterial populations (FAO/OIE/WHO 2004; Magouras et al. 2017; Tang et al. 2017). While there is a body of evidence demonstrating transfer of antimicrobial resistant bacteria between animals and people via direct contact or from food or environmental sources (Jordan, D. et al. 2011; Liu et al. 2016; McEwen, SA & Collignon 2018; Van Hoovels et al. 2006), there is little consensus regarding the overall effect antimicrobial use in animals has on human health. Notwithstanding this uncertainty, there is strong international support for the judicious use of antimicrobial agents in animals as a means of protecting public health and animal health (FAO/OIE/WHO 2004; United Nations 2016).

Many countries, particularly those in the European Union, have introduced restrictions or prohibitions on the use of important classes of antimicrobials in food animals, legislated antimicrobial reduction targets, undertaken benchmarking at the farm-level, and encouraged the adoption of antimicrobial stewardship programs (European Medicines Agency and European Food Safety Authority 2017). In Australia, a veterinary prescription is required for schedule 4 antimicrobial agents, and strict conditions for off-label or unregistered use of antimicrobials in food animals has been legislated for decades. The United States has recently introduced restrictions on the use of medically important antimicrobial agents in feed and now require greater veterinary oversight in the treatment of food animals. Similarly, in Canada, all medically important antimicrobial agents now require a veterinary prescription.

Restrictions on the use of certain antimicrobial classes in food animals have been reported to result in a reduction in antimicrobial resistance levels in those species (Aarestrup, FM et al. 2001; Bengtsson & Wierup 2006). For example, when the European Commission banned the use of avoparcin in animals in 1997, there was a marked reduction in the prevalence of vancomycin-resistant enterococci (VRE) in poultry faecal samples. Demark reported a decrease in VRE prevalence in poultry samples from over 80% in 1995, to less than 5% in 1998 (Aarestrup, F 2015), and the Netherlands reported a decrease from 80% to 31% in the two years between 1997-1999 (van den Bogaard, Bruinsma & Stobberingh 2000). In Australia, fluoroquinolones and fourth-generation cephalosporins have never been registered for use in

livestock, and consequently, bacterial resistance to these antimicrobial classes in livestock has not been reported (Cheng et al. 2012). When Canadian chicken producers voluntarily withdrew from using ceftiofur in 2005, the prevalence of ceftiofur resistance in *Salmonella* Heidelberg isolated from retail chicken meat dropped from over 60% to 7% by 2006. When ceftiofur use was partially reinstated in 2007, the prevalence of ceftiofur resistance in *Salmonella* Heidelberg strains increased to 18% by 2008 (Dutil et al. 2010). However, restrictions on antimicrobial use on their own may not eliminate resistant genes from an animal population, with recent studies demonstrating that for some antimicrobial agents such as avoparcin and ceftiofur, low-level prevalence of organisms resistant to these drugs can remain in a population of animals over time (Abraham et al. 2018; DANMAP 2017). Consequently, restriction or reduction in the use of an antimicrobial agent does not inevitably lead to the complete elimination of resistance (EMA/AMEG 2019).

Most national surveillance programs do not collect antimicrobial usage data from companion animals, except for a small number of countries, including Denmark and Sweden (DANMAP 2017; Swedres-Svarm 2017). Consequently, it is almost impossible to determine the extent of antimicrobial use and the potential effects this use has on the development of resistance in companion animals (Guardabassi, Schwarz & Lloyd 2004; Rushton 2015). Given there is limited legislative oversight, and a reported higher propensity to use antimicrobials of critical importance to humans in companion animal medicine, it is imperative that data is collected on the extent of antimicrobial use this sector. For example, Buckland et al. (2016) reported in their UK study of 374 small animals veterinary clinics that of all antimicrobial events described, 60% of events in dogs and 81% of events in cats were prescribed antimicrobial agents classified as critically important to human health. Other studies have also described a reliance on critically important antimicrobial agents in companion animals (Barber, Miller & McNamara 2003; Guardabassi, Schwarz & Lloyd 2004; Murphy et al. 2012; Smith et al. 2018). In Australia, it was reported that 18% of antimicrobial events used fluoroquinolones to treat dogs empirically, and 16% of antimicrobial events used a third-generation cephalosporin (i.e.,

cefovecin) for empirical treatment of cats (Hardefeldt et al. 2017). The study by Hardefeldt et al. (2017) also reported rates of use of critically important antimicrobial agents were substantially increased in pets with chronic conditions. Given direct contact is considered a likely transmission method of resistant bacteria between humans and pets, capturing usage data could be of enormous benefit in understanding the epidemiology of localised antimicrobial resistance spread, and the development of interventions and stewardship programs that aim to minimise the use of critically important antimicrobial agents in pets (Guardabassi 2013; Tang et al. 2017)

Information on antimicrobial use in food animals is not readily available in most countries, so the quantity and type of antimicrobial agents used in each sector are mostly unknown. For instance, in 2017, just 107 member countries were able to contribute quantitative antimicrobial use data to the OIE global database for the monitoring of antimicrobial agents, with most information limited to sales and import data (OIE 2017). In Australia, antimicrobial use data in animals are limited to nationally aggregated sales data with little other accompanying information (Australian Pesticides and Veterinary Medicines Authority 2014). Thus, alternative sources for collecting antimicrobial use data in food animals is needed in Australia. Few countries, other than Denmark and the Netherlands have implemented nation-wide automated monitoring systems (DANMAP 2017; Stege et al. 2003). These systems collect clinic and farm-level data, which is far more useful for developing effective interventions to manage antimicrobial use in animals. Other software-based monitoring systems such as VetCompass and SAVSNET, have been shown to be minimally intrusive in the collection of prescription-level data from small animal practices, however both require substantial resource allocation to extract and analyse data given the absence of standardisation of veterinary record-keeping (Buckland et al. 2016; Singleton et al. 2018).

Perhaps the most practical alternative to automated systems is the structured questionnaire, where point-prevalence data obtained from veterinarians and producers can be used for detailed analysis of antimicrobial use by species, production type, age class, and

disease syndromes. While questionnaires have well-recognised limitations, they are a proven method for data collection in the scientific literature (Bowling 2005). Questionnaires can be efficient, standardised, affordable, confidential, time-flexible, and adaptable for use in multiple species and over-time (Jordan, D. et al. 2009; WHO 2013). For food animals, the cooperation of farmers and veterinarians is critical to obtaining accurate antimicrobial use data, particularly given the complex issues and sensitivities surrounding the quantity, types, and reasons for use. Inevitably, multi-stakeholder involvement will be required to encourage farmers and veterinarians to share antimicrobial use data in food animals voluntarily. Engagement with multiple stakeholders can lead to increased complexity in the planning, execution, and analysis of results, and thereby potentially impact on the quality of inferences arising from the survey.

The interpretation of antimicrobial use data is challenging when there are several metrics used to quantify the data, and the information requirements of stakeholders are very different. Despite the efforts of national surveillance programs, it is widely recognised that the reporting of antimicrobial sales data is of limited benefit (Cameron, A & McAllister 2016; Guardabassi, Schwarz & Lloyd 2004; Rushton 2015; Silley, Simjee & Schwarz 2012). Silley, Simjee and Schwarz (2012) contend that between-country comparisons on antimicrobial use per species based on the tonnage of antimicrobial agents sold are misleading and that these data should never be used as proof of causality between animal use and resistance trends in people. However, others appear content to draw such conclusions, notwithstanding the limitations in the quality and accuracy of the data (Chantziaras et al. 2014; ECDC/EFSA/EMA 2015; Tang et al. 2017). Until we can reliably, accurately, and routinely collect antimicrobial use data at the herd-level, interpretation of the data should be limited.

The case for enhanced surveillance of antimicrobial resistance in animals

The term efficiency refers to the ability to accomplish a task with a minimum of resources, saving money, time, and labor. As such, it is essential to consider whether efficiencies can be identified in the conduct of antimicrobial resistance surveillance without compromising program objectives (which may not be sufficiently detailed in the first instance). Many of the challenges associated with surveillance of animals are yet to be overcome. Existing national surveillance programs have evolved from a strong microbiological approach to surveillance where the emphasis is placed on measuring the attributes of the individual isolate rather than defining the status of the broader population. In these programs, relatively small numbers of bacterial isolates per host species are tested against many antimicrobial agents. While this strategy yields excellent information regarding the resistance status of each isolate evaluated (high internal validity), it is an expensive approach to conducting surveillance in animals, has questionable external validity, may not be fit for all intended purposes when applied to surveillance in food animals (i.e. a focus at the population level), or is well suited to all countries, especially those with limited budgets.

An aspect of antimicrobial resistance surveillance missing from most national programs is the meaningfulness of the data to the livestock sector. Surveillance objectives and interventions are focused on public health outcomes without providing many benefits to participating livestock sectors, such as managing endemic bacterial diseases of livestock. Moreover, few programs report resistance and antimicrobial use data relevant to companion animals, further eroding opportunities to address antimicrobial resistance issues in animals adequately. The expansion of surveillance programs to evaluate bacterial pathogens from animals would have a positive effect on the implementation of strategies which aim to contain antimicrobial resistance in animals and provide veterinarians with the necessary information to optimise antimicrobial use.

Scope and aims of this thesis

This thesis presented an opportunity to identify innovative solutions to address issues associated with the quality of data arising from national surveillance programs for antimicrobial resistance. Questions raised and discussed in this thesis included:

1. *Is the disc diffusion assay accurate for use in a national surveillance program?*

There is an opportunity to collect susceptibility data from veterinary laboratories for use in national surveillance provided results from disc diffusion are comparable to broth microdilution. In this thesis, I evaluated the accuracy of disc diffusion relative to broth microdilution for two important pathogens of animals – *E. coli* and *S. pseudintermedius* (Chapters 2 and 3). The overlap between these two chapters is only partial since Chapter 3 expands on the methodology of its predecessor by including the evaluation of phenotypic assays against genetic approaches for detecting the presence of resistance determinants.

2. *How precise is the disc diffusion assay in veterinary diagnostic laboratories?*

Understanding measurement imprecision (variability) in a diagnostic test is critical for interpretation of results. Standardisation of protocols is necessary to ensure data can be repeated by different technicians either within the same laboratory or reproduced at different laboratories. It is essential for laboratories participating in national surveillance to adopt standardised protocols when performing the disc diffusion assay. Research into the repeatability and reproducibility of disc diffusion testing in Australian veterinary diagnostic laboratories will validate if it is possible to source surveillance data from laboratories (Chapter 4).

3. *Is robotic technology possible for the evaluation of antimicrobial resistance in large numbers of commensal bacteria?*

Current surveillance approaches suffer from very small sample sizes. The advantage of larger sample sizes is that it overcomes serious design weakness related to inadequate coverage of animal populations and generates more precise estimates of prevalence. If laboratory capacity is increased through the adoption of efficient testing methods, there is scope to increase the number of isolates appraised in surveillance. Furthermore, evaluation of the optimal number of antimicrobial agents to be included in a panel will reduce costs and may free up resources to evaluate more isolates. While it was intended that this thesis undertake a pilot study using high-throughput robotic technologies, the robotic equipment was not available in time for research to take place. This remains an important area of research to explore efficiency gains in surveillance.

4. *How well do questionnaires perform as part of a stakeholder-driven approach to collect farm-level data on antimicrobial use*?

Data based on antimicrobial sales is of limited value in the design of interventions to optimise antimicrobial use in livestock. Capturing antimicrobial usage data and information on stewardship practices at the farm-level will help identify factors that contribute to the persistence of resistant bacteria. However, the cooperation of herd-owners and veterinarians is critical to obtaining such data given the complex issues that exist involving specific antimicrobial agents, diseases, and treatment regimes. Herd-level data also benefit livestock producers and veterinarians in the development of prescribing guidelines and stewardship principles (Chapter 5).

References

Aarestrup, F. (2015) The livestock reservoir for antimicrobial resistance: a personal view on changing patterns of risks, effects of interventions and the way forward. *Philosophical Transactions of the Royal Society B-Biological Sciences*, 370(1670).

Aarestrup, F. M., Seyfarth, A. M., Emborg, H. D., Pedersen, K., Hendriksen, R. S. & Bager, F. (2001) Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. *Antimicrobial Agents and Chemotherapy*, 45(7), 2054-2059.

Abraham, S., Kirkwood, R. N., Laird, T., Saputra, S., Mitchell, T., Singh, M., Linn, B., Abraham, R. J., Pang, S., Gordon, D. M., Trott, D. J. & O'Dea, M. (2018) Dissemination and persistence of extended-spectrum cephalosporin-resistance encoding IncI1-blaCTXM-1 plasmid among Escherichia coli in pigs. *ISME J*, 12(10), 2352-2362.

Australian Chicken Meat Federation (2018) *Surveillance for antimicrobial resistance in enteric commensals and pathogens in Australian meat chickens. October 2018*, 2018.

Australian Commission on Safety and Quality in Health Care (2019) *CARAlert update 10: 1 November 2018–31 December 2018.* Sydney: ACSQHC; 2019

Australian Pesticides and Veterinary Medicines Authority (2014) *Quantity of Antimicrobial Products Sold for Veterinary Use in Australia: July 2005 to June 2010*, 2014.

Australian Strategic and Advisory Group on Antimicrobial Resistance (ASTAG) (2018) *Importance Ratings and Summary of Antibacterial Uses in Human and Animal Health in Australia, Version 1.0* Canberra, Australia: Commonwealth of Australia.

Barber, D. A., Miller, G. Y. & McNamara, P. E. (2003) Models of antimicrobial resistance and foodborne illness: examining assumptions and practical applications. *J Food Prot*, 66(4), 700-9.

Barlow, R. S., McMillan, K. E., Duffy, L. L., Fegan, N., Jordan, D. & Mellor, G. E. (2015) Prevalence and Antimicrobial Resistance of Salmonella and Escherichia coli from Australian Cattle Populations at Slaughter. *Journal of Food Protection*, 78(5), 912-920.

Barnhart, H. X., Haber, M. J. & Lin, L. I. (2007) An overview on assessing agreement with continuous measurements. *J Biopharm Stat*, 17(4), 529-69.

Bartlett, J. W. & Frost, C. (2008) Reliability, repeatability and reproducibility: analysis of measurement errors in continuous variables. *Ultrasound in Obstetrics & Gynecology*, 31(4), 466-475.

Barton, M. & Wilkins, J. (2001) *Antibiotic resistance in bacteria isolated from poultry: A report for the Rural Industries Research and Development Corporation*.

Bax, R., Bywater, R., Cornaglia, G., Goossens, H., Hunter, P., Isham, V., Jarlier, V., Jones, R., Phillips, I., Sahm, D., Senn, S., Struelens, M., Taylor, D. & White, A. (2001) Surveillance of antimicrobial resistance - what, how and whither? *Clinical Microbiology and Infection*, 7(6), 316-325.

Benedict, K. M., Gow, S. P., Checkley, S., Booker, C. W., McAllister, T. A. & Morley, P. S. (2013) Methodological comparisons for antimicrobial resistance surveillance in feedlot cattle. *BMC Vet Res*, 9, 216.

Benedict, K. M., Gow, S. P., McAllister, T. A., Booker, C. W., Hannon, S. J., Checkley, S. L., Noyes, N. R. & Morley, P. S. (2015) Antimicrobial Resistance in Escherichia coli Recovered from Feedlot Cattle and Associations with Antimicrobial Use. *PLoS One*, 10(12), e0143995.

Bengtsson, B. & Wierup, M. (2006) Antimicrobial resistance in Scandinavia after ban of antimicrobial growth promoters. *Anim Biotechnol*, 17(2), 147-56.

Bowling, A. (2005) Mode of questionnaire administration can have serious effects on data quality. *J Public Health (Oxf)*, 27(3), 281-91.

Brunden, M. N., Zurenko, G. E. & Kapik, B. (1992) Modification of the error-rate bounded classification scheme for use with two MIC break points. *Diagn Microbiol Infect Dis*, 15(2), 135-40.

Buckland, E. L., O'Neill, D., Summers, J., Mateus, A., Church, D., Redmond, L. & Brodbelt, D. (2016) Characterisation of antimicrobial usage in cats and dogs attending UK primary care companion animal veterinary practices. *Vet Rec*, 179(19), 489.

Cameron, A. & McAllister, T. A. (2016) Antimicrobial usage and resistance in beef production. *J Anim Sci Biotechnol*, 7, 68.

Cameron, A. R. (2012) The consequences of risk-based surveillance: Developing output-based standards for surveillance to demonstrate freedom from disease. *Prev Vet Med*, 105(4), 280-6.

Caprioli, A., Busani, L., Martel, J. L. & Helmuth, R. (2000) Monitoring of antimicrobial agent resistance in bacteria of animal origin: epidemiological and microbiological methodologies. *International Journal of Antimicrobial Agents*, 14(4), 295-301.

Chantziaras, I., Boyen, F., Callens, B. & Dewulf, J. (2014) Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. *J Antimicrob Chemother*, 69(3), 827-34.

Cheng, A. C., Turnidge, J., Collignon, P., Looke, D., Barton, M. & Gottlieb, T. (2012) Control of Fluoroquinolone Resistance through Successful Regulation, Australia. *Emerging Infectious Diseases*, 18(9), 1453-1460.

Craig, B. A. (2000) Modeling approach to diameter breakpoint determination. *Diagnostic Microbiology and Infectious Disease*, 36(3), 193-202.

DANMAP (2017) *DANMAP 2016 - Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. ISSN 1600-2032*.

Dargatz, D. A., Erdman, M. M. & Harris, B. (2017) A survey of methods used for antimicrobial susceptibility testing in veterinary diagnostic laboratories in the United States. *Journal of Veterinary Diagnostic Investigation,*, 29(5), 669-675.

Davison, H. C., Low, J. C. & Woolhouse, M. E. J. (2000) What is antimicrobial agent resistance and how can we measure it? *Trends in Microbiology*, 8(12), 554-559.

de Vet, H. C., Terwee, C. B., Knol, D. L. & Bouter, L. M. (2006) When to use agreement versus reliability measures. *J Clin Epidemiol*, 59(10), 1033-9.

DePalma, G., Turnidge, J. & Craig, B. A. (2017) Determination of disk diffusion susceptibility testing interpretive criteria using model-based analysis: development and implementation. *Diagn Microbiol Infect Dis*, 87(2), 143-149.

Didelot, X., Bowden, R., Wilson, D. J., Peto, T. E. A. & Crook, D. W. (2012) Transforming clinical microbiology with bacterial genome sequencing. *Nature Reviews Genetics*, 13(9), 601-612.

Dohoo, I., Martin, W. & Stryhn, H. (2009) *Veterinary Epidemiologic Research*, 2nd edition. Charlottetown, Prince Edward Island: VER Inc.

Dunlop, R. H., McEwen, S. A., Meek, A. H., Friendship, R. M., Black, W. D. & Clarke, R. C. (1999) Sampling considerations for herd-level measurement of faecal Escherichia coli antimicrobial resistance in finisher pigs. *Epidemiology and Infection*, 122(3), 485-496.

Dutil, L., Irwin, R., Finley, R., Ng, L. K., Avery, B., Boerlin, P., Bourgault, A. M., Cole, L., Daignault, D., Desruisseau, A., Demczuk, W., Hoang, L., Horsman, G. B., Ismail, J., Jamieson, F., Maki, A., Pacagnella, A. & Pillai, D. R. (2010) Ceftiofur resistance in Salmonella enterica serovar Heidelberg from chicken meat and humans, Canada. *Emerg Infect Dis*, 16(1), 48-54.

ECDC/EFSA/EMA (2015) ECDC/EFSA/EMA first joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals. *EFSA Journal*, 13(1), 4006-4006.

EMA/AMEG (2019) *Answer to the request from the European Commission for updating the scientific advice on the impact on publich health and animal health of the use of antibiotics in animals - Categorisation of antimicorbials. Draft*, 2019. Available online:

https://www.ema.europa.eu/en/documents/other/answer-request-european-commission-updating-scientific-advice-impact-public-health-animal-health-use_en.pdf [Accessed.

Enoe, C., Georgiadis, M. P. & Johnson, W. O. (2000) Estimation of sensitivity and specificity of diagnostic tests and disease prevalence when the true disease state is unknown. *Preventive Veterinary Medicine*, 45(1-2), 61-81.

European Food Safety Authority (2012) Technical specifications for the analysis and reporting of data on antimicrobial resistance in the European Union Summary Report. *EFSA Journal* 10(2):2587.

European Medicines Agency and European Food Safety Authority (2017) EMA and EFSA joint scientific opinion on measures to reduce the need to use antimicrobial agents in animal husbandry in the European Union, and the resulting impacts on food safety. *EFSA J*(15), 1–245.

Commission Implementing Decision on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria (2013/652/EU) (2013) Chapter Official Journal of the European Union.

FAO/OIE/WHO (2004) *Second joint FAO/OIE/WHO expert workshop on non-human antimicrobial usage and antimicrobial resistance: scientific assessment*.Geneva, Switzerland.

Fletcher-Lartey, S., Yee, M., Gaarslev, C. & Khan, R. (2016) Why do general practitioners prescribe antimicrobial agents for upper respiratory tract infections to meet patient expectations: a mixed methods study. *BMJ Open*, 6(10), e012244.

Fluit, A. C., van der Bruggen, J. T., Aarestrup, F. M., Verhoef, J. & Jansen, W. T. M. (2006) Priorities for antimicrobial agent resistance surveillance in Europe. *Clinical Microbiology and Infection*, 12(5), 410-417.

Franklin, A., Acar, J., Anthony, F., Gupta, R., Nicholls, T., Tamura, Y., Thompson, S., Threlfall, E. J., Vose, D., van Vuuren, M., White, D. G., Wegener, H. C. & Costarrica, M. L. (2001) Antimicrobial resistance: harmonisation of national antimicrobial resistance monitoring and surveillance programmes in animals and in animal-derived food. *Revue Scientifique Et Technique De L Office International Des Epizooties*, 20(3), 859-870.

Frickmann, H., Masanta, W. O. & Zautner, A. E. (2014) Emerging Rapid Resistance Testing Methods for Clinical Microbiology Laboratories and Their Potential Impact on Patient Management. *Biomed Research International*.

Gardner, I. A. & Greiner, M. (2000) Validation and application of diagnostic tests used in veterinary epidemiologic studies. *Preventive Veterinary Medicine*, 45(1/2), 1-162.

Gardner, I. A. & Greiner, M. (2006) Receiver-operating characteristic curves and likelihood ratios: improvements over traditional methods for the evaluation and application of veterinary clinical pathology tests. *Veterinary Clinical Pathology*, 35(1), 8-17.

Gart, J. J. & Buck, A. A. (1966) Comparison of a screening test and a reference test in epidemiologic studies. II. A probabilistic model for the comparison of diagnostic tests. *Am J Epidemiol*, 83(3), 593-602.

GERM-VET (2018) *Report on the resistance monitoring study 2016: Resistance situation in clinically important animal pathogenic bacteria*.Federal Office for Consumer Protection and Food Safety (BVL), Berlin.

Government of Canada (2017) *Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) 2015 Annual Report. ISSN:1925-9859.* Guelph, Ontario: Available online: http://publications.gc.ca/collections/collection_2017/aspc-phac/HP2-4-2015-eng.pdf [Accessed 21/2/2018].

Greiner, M. & Gardner, I. A. (2000) Epidemiologic issues in the validation of veterinary diagnostic tests. *Preventive Veterinary Medicine*, 45(1-2), 3-22.

Greiner, M., Pfeiffer, D. & Smith, R. D. (2000) Principles and practical application of the receiver-operating characteristic analysis for diagnostic tests. *Preventive Veterinary Medicine*, 45(1-2), 23-41.

Guardabassi, L. (2013) Sixty years of antimicrobial use in animals: what is next? *Vet Rec*, 173(24), 599-603.

Guardabassi, L., Schwarz, S. & Lloyd, D. H. (2004) Pet animals as reservoirs of antimicrobial-resistant bacteria. *J Antimicrob Chemother*, 54(2), 321-32.
Hardefeldt, L. Y., Holloway, S., Trott, D. J., Shipstone, M., Barrs, V. R., Malik, R., Burrows, M., Armstrong, S., Browning, G. F. & Stevenson, M. (2017) Antimicrobial Prescribing in Dogs and Cats in Australia: Results of the Australasian Infectious Disease Advisory Panel Survey. *Journal of Veterinary Internal Medicine*, 31(4), 1100-1107.
Hardefeldt, L. Y., Marenda, M., Crabb, H., Stevenson, M. A., Gilkerson, J. R., Billman-Jacobe, H. & Browning, G. F. (2018) Antimicrobial susceptibility testing by Australian veterinary diagnostic laboratories. *Australian Veterinary Journal*, 96(4), 142-146.
Hoinville, L. J. (2011) Animal Health Surveillance Terminology: Final report from Pre-ICAHS Workshop (Version 1.2). *In:* Hoinville, L. (ed.) *International Conference on Animal Health Surveillance.* https://www.fp7-risksur.eu/sites/default/files/partner_logos/icahs-workshop-2011_surveillance_tewrminology_report_V1.2.pdf. Accessed 31/08/2015.
Hoinville, L. J., Alban, L., Drewe, J. A., Gibbens, J. C., Gustafson, L., Hasler, B., Saegerman, C., Salman, M. & Stark, K. D. (2013) Proposed terms and concepts for describing and evaluating animal-health surveillance systems. *Prev Vet Med*, 112(1-2), 1-12.
Hombach, M., Jetter, M., Blochliger, N., Kolesnik-Goldmann, N. & Bottger, E. C. (2017) Fully automated disc diffusion for rapid antimicrobial agent susceptibility test results: a proof-of-principle study. *Journal of Antimicrobial Chemotherapy,*, 72(6), 1659-1668.
Hombach, M., Zbinden, R. & Bottger, E. C. (2013) Standardisation of disk diffusion results for antimicrobial agent susceptibility testing using the sirscan automated zone reader. *BMC Microbiology,*, 13, 225.
Humphry, R. W., Evans, J., Webster, C., Tongue, S. C., Innocent, G. T. & Gunn, G. J. (2018) An empirical comparison of isolate-based and sample-based definitions of antimicrobial resistance and their effect on estimates of prevalence. *Prev Vet Med*, 150, 143-150.
Idelevich, E. A., Becker, K., Schmitz, J., Knaack, D., Peters, G. & Kock, R. (2016) Evaluation of an automated system for reading and interpreting disk diffusion antimicrobial susceptibility testing of fastidious bacteria. *PLoS One*, 11(7), e0159183.
ISO (1994) Accuracy (Trueness and Precision) of Measurement Methods and Results - Part 1: General Principles and Definitions (5725-1). Geneva, Switzerland: ISO.
ISO (2006) Clinical Laboratory Testing and In Vitro Diagnostic Test Systems. Susceptibility Testing of Infectious Agents and Evaluation of Performance of Antimicrobial Susceptibility Test Devices. *Part 1: Reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases. 20776-1* Geneva, Switzerland: International Organisation for Standardisation.
Jordan, D. (2003) Surveillance for antimicrobial agent resistant Escherichia coli in food animals. *Communicable diseases intelligence quarterly report*, 27 Suppl, S117-20.
Jordan, D., Chin, J. J., Fahy, V. A., Barton, M. D., Smith, M. G. & Trott, D. J. (2009) Antimicrobial use in the Australian pig industry: results of a national survey. *Aust Vet J*, 87(6), 222-9.
Jordan, D., Simon, J., Fury, S., Moss, S., Giffard, P., Maiwald, M., Southwell, P., Barton, M. D., Axon, J. E., Morris, S. G. & Trott, D. J. (2011) Carriage of methicillin-resistant Staphylococcus aureus by veterinarians in Australia. *Aust Vet J*, 89(5), 152-9.
Kahlmeter, G., Brown, D. F., Goldstein, F. W., MacGowan, A. P., Mouton, J. W., Osterlund, A., Rodloff, A., Steinbakk, M., Urbaskova, P. & Vatopoulos, A. (2003) European harmonization of MIC breakpoints for antimicrobial susceptibility testing of bacteria. *J Antimicrob Chemother*, 52(2), 145-8.
Kidsley, A. K., Abraham, S., Bell, J. M., O'Dea, M., Laird, T. J., Jordan, D., Mitchell, P., McDevitt, C. A. & Trott, D. J. (2018) Antimicrobial Susceptibility of Escherichia coli and Salmonella spp. Isolates From Healthy Pigs in Australia: Results of a Pilot National Survey. *Frontiers in Microbiology*, 9.
Kottner, J., Audigé, L., Brorson, S., Donner, A., Gajewski, B. J., Hróbjartsson, A., Roberts, C., Shoukri, M. & Streiner, D. L. (2011) Guidelines for reporting reliability and agreement Studies (GRRAS). *Journal of Clinical Epidemiology*, 64(1), 96-106.

Kronvall, G., Giske, C. G. & Kahlmeter, G. (2011) Setting interpretive breakpoints for antimicrobial susceptibility testing using disk diffusion. *Int J Antimicrob Agents*, 38(4), 281-90.

Labricciosa, F. M., Sartelli, M., Correia, S., Abbo, L. M., Severo, M., Ansaloni, L., Coccolini, F., Alves, C., Melo, R. B., Baiocchi, G. L., Paiva, J. A., Catena, F. & Azevedo, A. (2018) Emergency surgeons' perceptions and attitudes towards antimicrobial agent prescribing and resistance: a worldwide cross-sectional survey. *World J Emerg Surg*, 13, 27.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K., Wertheim, H. F., Sumpradit, N., Vlieghe, E., Hara, G. L., Gould, I. M., Goossens, H., Greko, C., So, A. D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A. Q., Qamar, F. N., Mir, F., Kariuki, S., Bhutta, Z. A., Coates, A., Bergstrom, R., Wright, G. D., Brown, E. D. & Cars, O. (2013) Antimicrobial agent resistance-the need for global solutions. *Lancet Infect Dis*, 13(12), 1057-98.

Lehtopolku, M., Kotilainen, P., Puukka, P., Nakari, U. M., Siitonen, A., Eerola, E., Huovinen, P. & Hakanen, A. J. (2012) Inaccuracy of the disk diffusion method compared with the agar dilution method for susceptibility testing of Campylobacter spp. *Journal of Clinical Microbiology,*, 50(1), 52-6.

Lestari, E. S., Severin, J. A., Filius, P. M. G., Kuntaman, K., Duerink, D. O., Hadi, U., Wahjono, H., Verbrugh, H. A. & Study Grp Antimicrobial, R. (2008) Comparison of the accuracy of disk diffusion zone diameters obtained by manual zone measurements to that by automated zone measurements to determine antimicrobial susceptibility. *Journal of Microbiological Methods*, 75(2), 177-181.

Lewis, D. (2002) Antimicrobial resistance surveillance: methods will depend on objectives. *J Antimicrob Chemother*, 49(1), 3-5.

Liu, Y. Y., Wang, Y., Walsh, T. R., Yi, L. X., Zhang, R., Spencer, J., Doi, Y., Tian, G., Dong, B., Huang, X., Yu, L. F., Gu, D., Ren, H., Chen, X., Lv, L., He, D., Zhou, H., Liang, Z., Liu, J. H. & Shen, J. (2016) Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis*, 16(2), 161-8.

Magouras, I., Carmo, L. P., Stark, K. D. C. & Schupbach-Regula, G. (2017) Antimicrobial Usage and -Resistance in Livestock: Where Should We Focus? *Front Vet Sci*, 4, 148.

Mather, A. E., Reeve, R., Mellor, D. J., Matthews, L., Reid-Smith, R. J., Dutil, L., Haydon, D. T. & Reid, S. W. (2016) Detection of Rare Antimicrobial Resistance Profiles by Active and Passive Surveillance Approaches. *PLoS One*, 11(7), e0158515.

Matuschek, E., Brown, D. F. J. & Kahlmeter, G. (2014) Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clinical Microbiology and Infection*, 20(4), O255-O266.

McEwen, S., Aarestrup, F. & Jordan, D. (2006) Monitoring of Antimicrobial Resistance in Animals: Principles and Practices, in FM, A. (ed), *Antimicrobial Resistance in Bacteria of Animal Origin*. Washington DC: ASM Press.

McEwen, S. A. & Collignon, P. J. (2018) Antimicrobial Resistance: a One Health Perspective. *Microbiol Spectr*, 6(2).

Medeiros, A. A. & Crellin, J. (2000) Evaluation of the Sirscan automated zone reader in a clinical microbiology laboratory. *Journal of Clinical Microbiology,*, 38(4), 1688-93.

Murphy, C. P., Reid-Smith, R. J., Boerlin, P., Weese, J. S., Prescott, J. F., Janecko, N. & McEwen, S. A. (2012) Out-patient antimicrobial drug use in dogs and cats for new disease events from community companion animal practices in Ontario. *Can Vet J*, 53(3), 291-8.

Murray, P. R., Zeitinger, J. R. & Krogstad, D. J. (1982) Reliability of disc diffusion susceptibility testing. *Infection Control and Hospital Epidemiology*, 3(3), 230-7.

NORM/NORM-VET (2017) *Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway*.Tromsø / Oslo.

O'Neil, J. (2016) *Tackling drug-resistant infections globally: Final report and recommendations*.London, UK.

O'Neill, J. (2014) *Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations*.London, UK.

OIE (2015a) *OIE List of antimicrobials of veterinary importance*. *Resolution 26. Combating Antimicrobial Resistance and Promoting the Prudent Use of Antimicrobial Agents in Animals. 83rd General Assembly* (2015b) Resolution 26, Chapter Paris: http://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/AMR/A_RESO_AMR_2015.pdf.

OIE (2016) *Chapter 6.7. Harmonisation of national antimicrobial resistance surveillance and monitoring programs.* OIE.

OIE (2017) OIE Annual report on the use of antimicrobial agents intended for use in animals: better understanding of the global situation. 2nd Report. Paris: OIE.

OIE (2018a) Chapter 1.1.6. Principles and Methods of Validation of Diagnostic Assays for Infectious Diseases. Adopted 2013, *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 7th Edition*

OIE (2018b) Chapter 2.1.1 Laboratory Methodologies for Bacterial Antimicrobial Susceptibility Testing. Adopted 2012, *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 7th Edition*

Persoons, D., Bollaerts, K., Smet, A., Herman, L., Heyndrickx, M., Martel, A., Butaye, P., Catry, B., Haesebrouck, F. & Dewulf, J. (2011) The Importance of Sample Size in the Determination of a Flock-Level Antimicrobial Resistance Profile for Escherichia coli in Broilers. *Microbial Drug Resistance*, 17(4), 513-519.

Rempel, O., Pitout, J. D. D. & Laupland, K. B. (2011) Antimicrobial resistance surveillance systems: Are potential biases taken into account? *Canadian Journal of Infectious Diseases & Medical Microbiology*, 22(4), E24-E28.

RESAPTH (2017) *French surveillance network for antimicrobial resistance in pathogenic bacteria of animal origin: 2015 Annual Report*.Lyon et Ploufragan-Plouzané.

Rogan, W. J. & Gladen, B. (1978) Estimating prevalence from the results of a screening test. *Am J Epidemiol*, 107(1), 71-6.

Rushton, J. (2015) Anti-microbial Use in Animals: How to Assess the Trade-offs. *Zoonoses Public Health*, 62 Suppl 1, 10-21.

Sahibzada, S., Abraham, S., Coombs, G. W., Pang, S., Hernandez-Jover, M., Jordan, D. & Heller, J. (2017) Transmission of highly virulent community-associated MRSA ST93 and livestock-associated MRSA ST398 between humans and pigs in Australia. *Scientific Reports*, 7.

Salman, M. D. (2003) Surveillance and Monitoring Systems for ANimal Health Programs and Disease Surveys, in Salman, M. D. (ed), *Animal disease surveillance and survey systems: methods and applications*, 1st edition. Ames, Iowa: Iowa State Press.

Schwartz, D. (2012) Prediction of lysine post-translational modifications using bioinformatic tools. *Essays Biochem*, 52, 165-77.

Schwarz, S., Silley, P., Simjee, S., Woodford, N., van Duijkeren, E., Johnson, A. P. & Gaastra, W. (2010) Assessing the antimicrobial susceptibility of bacteria obtained from animals. *Veterinary Microbiology*, 141(1-2), 1-4.

Shaban, R., Simon, G., Trott, D., Turnidge, J. & Jordan, D. (2014) *Surveillance and reporting of antimicrobial resistance and antibiotic usage in animals and agriculture in Australia*.Canberra: Australia, C. o.

Silley, P. (2012) Susceptibility testing methods, resistance and breakpoints: what do these terms really mean? *Revue Scientifique Et Technique-Office International Des Epizooties*, 31(1), 33-41.

Silley, P., Simjee, S. & Schwarz, S. (2012) Surveillance and monitoring of antimicrobial resistance and antimicrobial agent consumption in humans and animals. *Revue Scientifique Et Technique-Office International Des Epizooties*, 31(1), 105-120.

Singleton, D. A., Sanchez-Vizcaino, F., Arsevska, E., Dawson, S., Jones, P. H., Noble, P. J. M., Pinchbeck, G. L., Williams, N. J. & Radford, A. D. (2018) New approaches to pharmacosurveillance for monitoring prescription frequency, diversity, and co-

prescription in a large sentinel network of companion animal veterinary practices in the United Kingdom, 2014-2016. *Prev Vet Med*, 159, 153-161.

Smith, M., King, C., Davis, M., Dickson, A., Park, J., Smith, F., Currie, K. & Flowers, P. (2018) Pet owner and vet interactions: exploring the drivers of AMR. *Antimicrob Resist Infect Control*, 7, 46.

Stege, H., Bager, F., Jacobsen, E. & Thougaard, A. (2003) VETSTAT-the Danish system for surveillance of the veterinary use of drugs for production animals. *Prev Vet Med*, 57(3), 105-15.

Swedres-Svarm (2017) *Consumption of antibiotics and occurrence of antibiotic resistance in Sweden*.Solna/Uppsala ISSN 1650-6332.

Swets, J. A. (1988) MEASURING THE ACCURACY OF DIAGNOSTIC SYSTEMS. *Science*, 240(4857), 1285-1293.

Tang, K. L., Caffrey, N. P., Nobrega, D. B., Cork, S. C., Ronksley, P. E., Barkema, H. W., Polachek, A. J., Ganshorn, H., Sharma, N., Kellner, J. D. & Ghali, W. A. (2017) Restricting the use of antimicrobial agents in food-producing animals and its associations with antimicrobial agent resistance in food-producing animals and human beings: a systematic review and meta-analysis. *Lancet Planet Health*, 1(8), e316-e327.

Thacker, S. B., Qualters, J. R. & Lee, L. M. (2012) Public health surveillance in the United States: evolution and challenges. *MMWR Suppl*, 61(3), 3-9.

Thrusfield, M. V. (2007) *Veterinary Epidemiology*, Third edition. Oxford: Blackwell.

Toutain, P. L., Bousquet-Melou, A., Damborg, P., Ferran, A. A., Mevius, D., Pelligand, L., Veldman, K. T. & Lees, P. (2017) En Route towards European Clinical Breakpoints for Veterinary Antimicrobial Susceptibility Testing: A Position Paper Explaining the VetCAST Approach. *Front Microbiol*, 8, 2344.

Turnidge, J. & Paterson, D. L. (2007) Setting and revising antibacterial susceptibility breakpoints. *Clin Microbiol Rev*, 20(3), 391-408.

United Nations (2016) *At UN, global leaders commit to act on antimicrobial resistance. Collective effort to address a challenge to health, food security and development. Joint News Release, 21 September 2016.* New York: http://www.who.int/mediacentre/news/releases/2016/commitment-antimicrobial-resistance/en/.

USDA, N. A. R. M. S. (2017) *NARMS Integrated Report (2015).* Athens, GA: U.S.: Department of Agriculture, Agricultural Research Service.

van den Bogaard, A. E., Bruinsma, N. & Stobberingh, E. E. (2000) The effect of banning avoparcin on VRE carriage in The Netherlands. *J Antimicrob Chemother*, 46(1), 146-8.

Van Hoovels, L., Vankeerberghen, A., Boel, A., Van Vaerenbergh, K. & De Beenhouwer, H. (2006) First case of Staphylococcus pseudintermedius infection in a human. *J Clin Microbiol*, 44(12), 4609-12.

Vieira, A. R., Wu, S., Jensen, L. B., Dalsgaard, A., Houe, H., Wegener, H. C., Wong, D. M. A. L. F. & Emborg, H. D. (2008) Using data on resistance prevalence per sample in the surveillance of antimicrobial resistance. *Journal of Antimicrobial Chemotherapy*, 62(3), 535-538.

Wagner, B. A., Dargatz, D. A., Salman, M. D., Morley, P. S., Wittum, T. E. & Keefe, T. J. (2002) Comparison of sampling techniques for measuring the antimicrobial susceptibility of enteric Escherichia coli recovered from feedlot cattle. *American Journal of Veterinary Research*, 63(12), 1662-1670.

White, D. G., Acar, J., Anthony, F., Franklin, A., Gupta, B., Nicholls, T., Tamura, Y., Thompson, S., Threlfall, E. J., Vose, D., van Vuuren, M., Wegener, H. C. & Costarrica, M. L. (2001) Antimicrobial resistance: standardisation and harmonisation of laboratory methodologies for the detection and quantification of antimicrobial resistance. *Revue Scientifique Et Technique De L Office International Des Epizooties*, 20(3), 849-858.

WHO (2001) *WHO Global Strategy for Containment of Antimicrobial Resistance*.Geneva, Switzerland.

WHO (2013) *Integrated surveillance of antimicrobial resistance: guidance from a WHO Advisory Group*.Geneva, Switzerland.
WHO (2015) *Global Action Plan on Antimicrobial Resistance.* Geneva: http://www.wpro.who.int/entity/drug_resistance/resources/global_action_plan_eng.pdf.
WHO (2017a) *Global antimicrobial resistance surveillance system (GLASS) report: early implementation 2016-2017* [eBook]. Geneva: World Health Organization
WHO (2017b) Integrated Surveillance of Antimicrobial Resistance in Foodborne Bacteria: Application of a One Health Approach. Geneva: World Health Organization.

Chapter 2:

Relative Performance of Antimicrobial Susceptibility Assays on Clinical *Escherichia coli* Isolates from Animals

Contextual Statement

Phenotypic assays such as disc diffusion and broth microdilution are used in clinical and surveillance settings to determine the antimicrobial susceptibility of bacterial isolates. While broth microdilution is the preferred assay for surveillance, disc diffusion is commonly used in veterinary diagnostic laboratories. There is considerable scope to acquire disc diffusion data from veterinary laboratories for the surveillance of bacterial pathogens provided the results are comparable to broth microdilution. However, the performance of phenotypic assays is poorly understood, as they are used in veterinary diagnostic laboratories. This raises questions about the quality of information reported by national surveillance programs for antimicrobial resistance. Thus, the aim of this study was to evaluate the accuracy of disc diffusion relative to broth microdilution (the reference test) for clinical *Escherichia coli* isolates (n=994) derived from companion animals. In this study, conventional statistical methods are used to evaluate the accuracy of disc diffusion relative to broth microdilution, including the reporting of relative diagnostic sensitivity and specificity, likelihood ratio pairs, and receiver-operating characteristic (ROC) analysis.

Statement of Authorship

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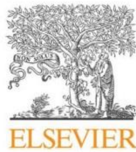

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Relative performance of antimicrobial susceptibility assays on clinical *Escherichia coli* isolates from animals

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ABSTRACT

The assessment of antimicrobial resistance in bacteria derived from animals is often performed using the disc diffusion assay. However broth-microdilution is the preferred assay for national antimicrobial resistance surveillance programs. This study aimed to evaluate the accuracy of disc diffusion relative to broth-microdilution across a panel of 12 antimicrobials using data from a collection of 994 clinical *Escherichia coli* isolates from animals. Disc diffusion performance was evaluated by diagnostic sensitivity, specificity, likelihood ratio pairs and receive-operating characteristic (ROC) analysis. Data was dichotomised using CLSI susceptible and resistant clinical breakpoints. In addition, disc diffusion breakpoints produced using diffusion Breakpoint Estimation Testing Software (dBETS) were evaluated. Analysis revealed considerable variability in performance estimates for disc diffusion susceptible and resistant breakpoints (AUC ranges: 0.78–0.99 and 0.92–1.0, respectively) across the panel of antimicrobials. Ciprofloxacin, tetracycline, and ampicillin estimates were robust across both breakpoints, whereas estimates for several antimicrobials including amoxicillin-clavulanic acid, cefoxitin and gentamicin were less favourable using susceptible breakpoints. Overall performance estimates were moderately improved when dBETS susceptible breakpoints were applied. For most antimicrobials, disc diffusion was accurate at predicting resistance of clinical *E. coli* from animals that could otherwise be determined by broth-microdilution. While disc diffusion is suboptimal for assessing the proportion of fully susceptible isolates for some drugs, sensitivity and specificity estimates provided here allow for the use of standard formula to correct this. For this reason, disc diffusion has applicability in national surveillance provided the performance of the assay is taken into account.

1. Introduction

The emergence and spread of bacteria resistant to multiple antimicrobials including 'last-line of defence' drugs is a critical threat to the well-being of humans, animals and the environment. Strong international consensus for global action on antimicrobial resistance (AMR) has been established within the United Nations General Assembly (United Nations, 2016) and international agencies responsible for human health, animal health and agriculture (OIE, 2015; WHO, 2015b). National surveillance programs are the cornerstone in global efforts to contain the spread of AMR (WHO, 2015a). Integrated national surveillance involving the coordinated collection of data on AMR in humans, animals and the environment is critical for detecting emerging forms of resistance and evaluating the success of policies designed to contain AMR (Laxminarayan et al., 2013).

Surveillance of AMR in animal-derived bacteria is typically focussed on commensal and zoonotic bacteria from food-producing animals rather than clinical isolates from diseased animals. While zoonotic

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Table 1
Disc diffusion and broth-microdilution interpretative criteria for twelve antimicrobials evaluated in this study and applied to 994 clinical *Escherichia coli* isolates derived from animals.

		Susceptible Breakpoints		Resistant Breakpoints		
Antimicrobial	Abbreviation	Disc diffusion zone diameter (mm)	Broth-microdilution MIC (µg/ml)	Disc diffusion zone diameter (mm)	Broth-microdilution MIC (µg/ml)	MIC range (µg/ml)
Amoxicillin-clavulanic acid	AMC	≥18[a]	≤8[a]	≤13[a]	≥32[a]	1.0–64
Amikacin	AMK	≥17[a]	≤16[a]	≤14[a]	≥64[a]	0.5–64
Ampicillin	AMP	≥17[a]	≤8[a]	≤13[a]	≥32[a]	1.0–128
Cephalothin	CEF	≥18[a]	≤8[a]	≤14[a]	≥32[a]	2.0–128
Ceftiofur	CFT	≥21[a]	≤2[a]	≤17[a]	≥8[a]	0.06–64
Ciprofloxacin	CIP	≥21[b]	≤1[b]	≤15[b]	≥4[b]	0.008–8
Cefovecin	CVN	≥23[c]	≤2[c]	≤19[c]	≥8[c]	0.12–128
Cefoxitin	FOX	≥18[b]	≤8[b]	≤14[b]	≥32[b]	1.0–128
Gentamicin	GEN	≥16[a]	≤2[a]	≤12[a]	≥8[a]	0.12–64
Imipenem	IPM	≥23[a]	≤1[a]	≤19[a]	≥4[a]	0.06–4
Trimethoprim-sulfamethoxazole	SXT	≥16[a]	≤2[a]	≤10[a]	≥4[a]	0.12–16
Tetracycline	TET	≥19[a]	≤4[a]	≤14[a]	≥16[a]	0.12–128

[a] Derived from CLSI VET01-S3.
[b] Derived from CLSI M100-S25.
[c] Cefovecin breakpoints based on manufacturer's recommendation.

Table 2
Diagnostic performance estimates of disc diffusion relative to broth-microdilution for 994 clinical *Escherichia coli* isolates from animals using CLSI susceptible and resistant breakpoints. DSe, diagnostic sensitivity; DSp diagnostic specificity; AUC, area under the curve. Exact 95% confidence intervals are given in Supplementary materials.

	Susceptible Breakpoint Estimates			Resistant Breakpoint Estimates		
Antimicrobial	Relative DSe	Relative DSp	AUC	Relative DSe	Relative DSp	AUC
Amoxicillin-clavulanic acid	0.23	0.99	0.82	0.79	0.99	0.98
Amikacin	NA	0.99	NA	NA	1.0	NA
Ampicillin	0.93	0.81	0.96	0.97	0.95	0.98
Cephalothin	0.70	0.81	0.82	0.75	0.98	0.92
Ceftiofur	0.84	0.99	0.94	0.94	0.99	0.98
Ciprofloxacin	0.96	1.0	0.99	0.99	1.0	1.0
Cefovecin	0.67	0.96	0.87	0.88	0.99	0.97
Cefoxitin	0.33	1.0	0.78	0.83	0.99	0.97
Gentamicin	0.50	0.99	0.82	0.92	1.0	0.97
Imipenem	NA	0.99	NA	NA	1.0	NA
Trimethoprim-sulfamethoxazole	0.70	0.99	0.93	0.72	0.99	0.94
Tetracycline	0.93	0.98	0.97	0.95	0.99	0.98

NA, not available due to insufficient data for the analysis.

bacteria such as *Salmonella* spp. and *Campylobacter* spp. pose the greatest health threat to humans, commensal organisms of the gastrointestinal tract such as *Escherichia coli* are also considered high-risk for the transmission of antimicrobial resistance genes to human bacteria via food products (Shaban et al., 2014). A barrier to achieving comprehensive surveillance of all AMR risks in animals is the acquisition of data from a sufficient number of clinical isolates. This could be overcome by collecting antimicrobial assay results from veterinary laboratories either as minimum inhibitory concentration (MIC) from dilution-based assays or millimetres of zone diameter from diffusion-based assays. The MIC is widely considered to be the superior measure for quantifying an isolate's susceptibility to antimicrobials (Turnidge and Paterson, 2007), and hence, broth-microdilution is the preferred susceptibility assay for national surveillance programs (ISO, 2006; OIE, 2017b). However, disc diffusion is often favoured by veterinary laboratories as it is affordable and readily customisable for a range of animal pathogens. There is considerable scope to merge susceptibility data acquired from disc diffusion from multiple laboratories into national surveillance provided the results are comparable to those from MIC assays.

The overall accuracy of disc diffusion relative to broth-microdilution remains inconclusive despite several previous studies having evaluated the assay's performance across a range of bacterial species and antimicrobials (Benedict et al., 2013; Hoelzer et al., 2011; Klement et al., 2005; Rhodes et al., 2014; Saini et al., 2011; Schumacher et al., 2001). This may be due to limitations of isolates entering such studies including small sample size, study validity (i.e. isolates are not obtained from an epidemiologically relevant population from which inferences can be drawn) and low prevalence of resistance to antimicrobials, particularly those that are critically important to humans. For instance, of those studies which include animal-derived *E. coli*, only Benedict et al. (2013) (n = 3362), Klement et al. (2005) (n = 231) and Rhodes et al. (2014) (n = 304) assessed more than 200 isolates. Many previous studies have also constrained the evaluation of test performance to descriptive measures such as observed agreement of dichotomous results, simple linear regression and error-rate bounding without considering modern statistical approaches that fully exploit the data to aid interpretation of test performance.

Inevitably the assessment of diagnostic test accuracy relies on the reference test (usually broth-microdilution) and the cut-point (or breakpoint) used to dichotomise the data. In the context of AMR, the *clinical* breakpoint may define full susceptibility (susceptible breakpoint), resistance (resistant breakpoint) or the non-susceptible population (i.e. the combination of resistant and intermediate isolates) based on available pharmacokinetic data. In the evaluation of disc diffusion performance, some studies have applied the resistant breakpoint (Benedict et al., 2013; Hoelzer et al., 2011) while others applied the susceptible breakpoint (Klement et al., 2005; Saini et al., 2011). Inevitably different breakpoints will yield different estimates of test accuracy, with a resultant trade-off between the two types of

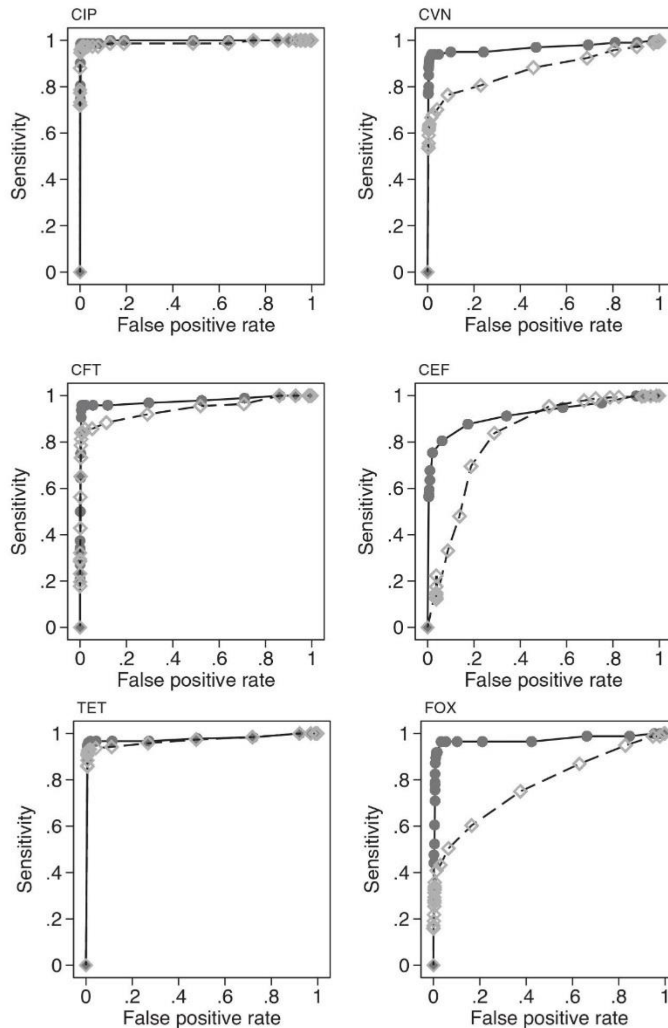

Fig. 1. ROC plots demonstrating overall performance of disc diffusion relative to broth-microdilution assays in clinical *Escherichia coli* isolates from animals for six antimicrobials. The black-closed-dot curve and the open-diamond-dash curve represent the dichotomisation at resistant and susceptible breakpoints respectively. CIP, ciprofloxacin; CVN, cefovecin; CFT, ceftiofur; CEF, cephalothin; TET, tetracycline; FOX, cefoxitin.

misclassification errors – false negatives and false positives. While both misclassification errors have consequences, false negatives (i.e. classified susceptible when truly resistant) are the least desired in the clinical setting. Given the breakpoint is crucial for overall assessment of test performance, inconsistency in the use of breakpoints to dichotomise data across studies is likely to also be a key factor in the reported variable performance of disc diffusion relative to MIC-based assays. This is particularly relevant when the diagnostic test is used for different purposes as is the case in the clinical setting versus broad-scale surveillance. The receiver-operating characteristic (ROC) analysis addresses this by estimating the overall diagnostic accuracy of tests with continuous outcomes across all potential breakpoints.

Therefore, the aim of this study was to develop a robust statistical approach to evaluate the accuracy of zone diameter measurements obtained by disc diffusion relative to MIC measurements obtained by broth-microdilution. The approach uses ROC analysis to summarise the relative accuracy of zone diameter measurements compared to MIC results (from the same isolates) across a large collection of clinical *E. coli* isolates from animals. Twelve antimicrobials relevant to animal health and public health were included for evaluation. For completeness, accuracy was evaluated using both susceptible and resistant clinical breakpoints recommended by the Clinical Laboratory Standards Institute (CLSI). In addition, new disc diffusion clinical breakpoints were produced using the model-based diffusion Breakpoint Estimation Testing Software (dBETS) and compared to CLSI breakpoints.

2. Methods

2.1. Isolate collection

Data used in this study were derived from the first nation-wide survey for antimicrobial resistance in veterinary pathogens, which took place between January 2013 and January 2014 with the cooperation of all veterinary diagnostic laboratories (n = 22) in Australia (Abraham et al., 2015). The data included disc diffusion and broth-microdilution results from 994 clinical *E. coli* isolates from canine (n = 510), feline (n = 338), equine (n = 28), and other species (n = 118), excluding food-producing animals.

Table 3
Estimates of likelihood ratios of disc diffusion relative to broth-microdilution for 994 clinical *Escherichia coli* isolates using CLSI susceptible and resistant breakpoints. LR^+, likelihood ratio of a positive test result; LR^-, likelihood ratio of a negative result. Exact 95% confidence intervals are given in the Supplementary materials.

	Susceptible breakpoint estimates		Resistant breakpoint estimates	
Antimicrobial	LR^+	LR^-	LR^+	LR^-
Amoxicillin-clavulanic acid	15.8	0.79	118.1	0.21
Amikacin	NA	NA	NA	NA
Ampicillin	4.8	0.09	21.0	0.03
Cephalothin	3.7	0.37	35.4	0.25
Ceftiofur	67.3	0.16	168.4	0.06
Ciprofloxacin	220.6	0.04	454.6	0.01
Cefovecin	17.2	0.34	131.2	0.12
Cefoxitin	61.8	0.67	124.9	0.18
Gentamicin	63.3	0.51	289.3	0.08
Imipenem	NA	NA	NA	NA
Trimethoprim-sulfamethoxazole	68.8	0.31	72.9	0.28
Tetracycline	53.5	0.07	154.4	0.05

NA, not available due to insufficient data for the analysis.

2.2. Antimicrobial susceptibility testing

E. coli isolates underwent disc diffusion and broth-microdilution testing according to CLSI VET01-A4 protocols (CLSI, 2013). The MIC results for the isolate collection were obtained from a previous study (Saputra et al., under review Vet Microbiol). Disc diffusion testing was performed independently and at a different point in time to when broth-microdilution testing occurred. Antimicrobial agents used in this study are listed in Table 1. The dataset was dichotomised for each antimicrobial and both assays using the susceptible and resistant clinical breakpoints specified in CLSI performance standards VET01-S3 (CLSI, 2015a) and M100-S25 (CLSI, 2015b) (Table 1). For dichotomisation using the susceptible clinical breakpoint, isolates clinically referred to as 'intermediate' or 'resistant' were collectively classified as 'non-susceptible'. For dichotomisation using the resistant clinical breakpoint, isolates were classified as 'susceptible' if their measurement value fell in the susceptible or intermediate range. Where animal-specific clinical breakpoints were unavailable or did not have corresponding MIC and zone diameter breakpoints, human clinical breakpoints were used as indicated. The exception was cefovecin as there were no CLSI clinical breakpoints available, so MIC and zone diameter susceptible and resistant breakpoints were used according to the manufacturer's recommendations. In this paper, unless otherwise specified, reference to susceptible and resistant MIC and zone diameter breakpoints refer to the CLSI recommended *clinical* breakpoints.

2.3. Statistical analysis

2.3.1. Relative diagnostic accuracy

The accuracy of disc diffusion classification relative to MIC (the reference method) was evaluated by estimating relative diagnostic sensitivity, diagnostic specificity, likelihood ratios of positive and negative results, and summarised using receiver-operating characteristic (ROC) analysis. MIC and zone diameters were compared using non-parametric ROC analysis since MIC data cannot be assumed to be normally distributed. For a given breakpoint, likelihood ratio pairs summarise how many times more (or less) likely a resistant isolate will be classified as resistant then an isolate that is fully susceptible. The likelihood ratio describes the direction and strength of evidence provided by a given test result. Details on likelihood ratios and area-under the ROC-curve (AUC) estimations are given elsewhere (Greiner and Gardner, 2000).

2.3.2. Agreement estimation

Observed agreement was calculated as the proportion of isolates with the same AMR clinical classification by disc diffusion and broth-microdilution (i.e. both test results were within the susceptible breakpoint range, or within the resistant breakpoint range). McNemar's mid-*p* test (Fagerland et al., 2013) was used to assess significance (two-tailed $p < 0.05$) in the extent of disagreement between the two tests. The mid-*p* version of the McNemar's test was used instead of the conventional McNemar's test as the count of discordant results between the two methods was often less than 25. Prevalence adjusted, bias adjusted kappa (PABAK) was calculated as a measure of agreement to adjust for imbalances caused by extreme prevalence and bias between tests (Byrt et al., 1993).

2.3.3. dBETS disc diffusion breakpoint values

The recently published diffusion Breakpoint Estimation Testing Software (dBETS) program (https://dbets.shinyapps.io/dBETS/, accessed 25 April 2017) was used to generate zone diameter susceptible and resistant clinical breakpoints for the antimicrobials evaluated in this dataset (DePalma et al., 2017). The dBETS program was used to apply spline-based probability models to account for disc diffusion assay variability, providing an advantage over commonly used methods such as the modified error-rate bounded method.

Data was imported from MS excel files into Stata version 14.1 (Stata Corporation, College Station, TX) for analysis. For each isolate and each of the 12 antimicrobials tested, the broth-microdilution and disc diffusion results were paired in wide format.

3. Results

For eleven antimicrobial agents, 994 paired observations on zone diameter by disc diffusion and MIC by broth-microdilution were available for analysis. For cefovecin, 948 paired observations were available. The overall performance of disc diffusion relative to broth-microdilution was very strong for ten antimicrobials (two antimicrobials were not evaluated due to insufficient data) at the resistant breakpoints (AUC range: 0.92–1.0) (Table 2). However at susceptible breakpoints, overall performance for all 12 antimicrobials was appreciably lower (AUC range: 0.78–0.99) (Table 2). At the susceptible breakpoint, sensitivity and specificity (reflected by AUC) varied across the antimicrobial panel, and was suboptimal for amoxicillin-clavulanic acid (AUC, 0.82), cephalothin (AUC, 0.82), cefoxitin (AUC, 0.78) and gentamicin (AUC, 0.82). Performance estimates for ciprofloxacin, trimethoprim-sulfamethoxazole and tetracycline were relatively unaffected by the choice of breakpoint (Table 2). AUC estimates could not be determined for amikacin and imipenem as the isolates were all susceptible by the reference method.

Visual comparison of ROC plots for ciprofloxacin, ceftiofur, cefovecin, ceftiofur, cephalothin, tetracycline and cefoxitin are presented in Fig. 1. Here, two ROC curves are plotted on each graph to demonstrate the accuracy of disc diffusion relative to broth-microdilution using the MIC susceptible and resistant breakpoints. For ciprofloxacin, ceftiofur, and tetracycline both susceptible and resistant ROC plots show near perfect test discrimination (both curves approach the top left corner of the graph). In contrast, cefovecin, cephalothin, and cefoxitin have higher levels of misclassification error (curves distant from the top left hand corner of the graph) (Fig. 1).

Table 2 shows that when resistant breakpoints were applied, relative specificity was high across all antimicrobials (range, 0.95–1.0) while relative sensitivity was variable (range, 0.72–0.99). When susceptible breakpoints were applied, relative specificity (range, 0.81 –1.0) and sensitivity (range, 0.23–0.96) estimates were notably more variable. By these criteria, disc diffusion performed poorly for several antimicrobials especially amoxicillin-clavulanic acid, cefoxitin and gentamicin. When interpreting a positive disc diffusion result, using resistant breakpoints provided stronger evidence (large LR^+) compared

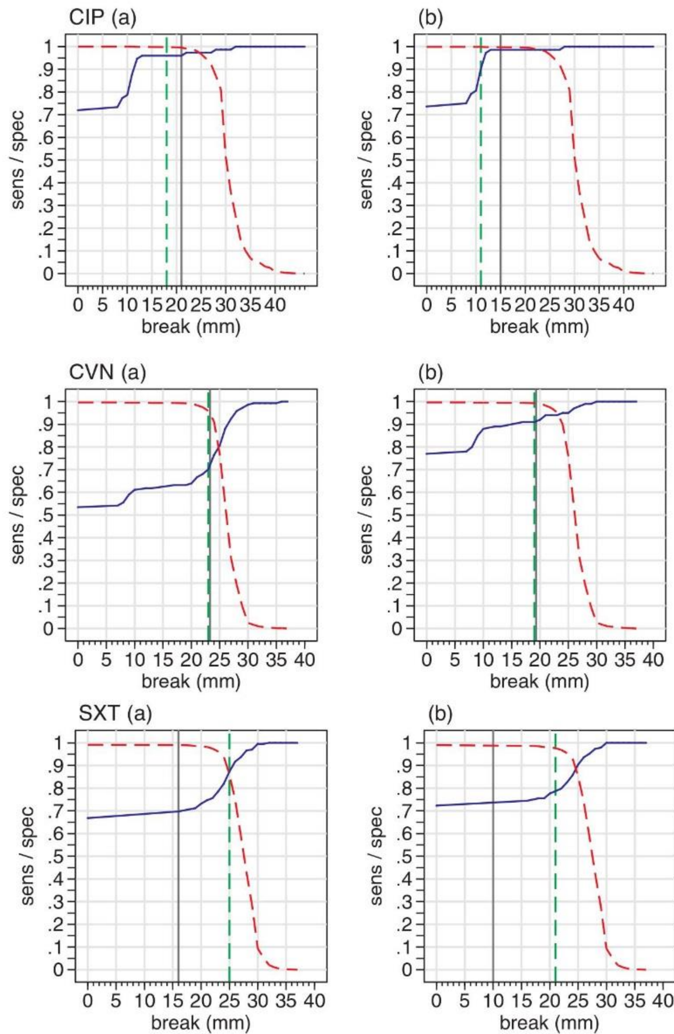

Fig. 2. Two-graph ROC (TG-ROC) plots of disc diffusion performance relative to broth-microdilution for ciprofloxacin (CIP), cefovecin (CVN), and trimethoprim-sulfamethoxazole (SXT). The TG-ROC curves for (a) susceptible and (b) resistant breakpoints are represented in the left and right column (a) and (b) respectively. Relative sensitivity (blue solid line), relative specificity (red dash line), CLSI breakpoint (black solid line), and dBETS breakpoint (green-dash line) are plotted on each graph. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to susceptible breakpoints (LR^+ ranges: 21–454.6 and 3.7–220.6, respectively) (Table 3). Similarly, the evidence provided by negative disc diffusion results were stronger (small LR^-) when using resistant breakpoints compared to susceptible breakpoints (LR^- ranges: 0.01–0.28 and 0.04–0.79, respectively). Evidence from a positive disc diffusion result was weakest for cephalothin and ampicillin (lowest LR^+) and strongest for ciprofloxacin (highest LR^+) regardless of the breakpoint (Table 3). Evidence from a negative disc diffusion result was weakest for amoxicillin-clavulanic acid (highest LR^-) and strongest for ciprofloxacin (lowest LR^-) (Table 3).

Two-graph receiver-operating characteristic (TG-ROC) plots for disc diffusion relative to broth-microdilution shows the impact of breakpoint on sensitivity and specificity and hence the level of misclassification error (Fig. 2). Sensitivity and specificity are equal at the point where the two lines intersect on the TG ROC plot, however the point of intersection does not always equate to the optimal breakpoint since the cost of misclassification errors almost always differs. CLSI and dBETS zone diameter breakpoints are plotted for comparison. For ciprofloxacin, CLSI and dBETS susceptible and resistant breakpoints correspond to almost perfect specificity with optimal sensitivity estimates (Tables 2 and 5). Similarly using both approaches, breakpoints for cefovecin and trimethoprim-sulfamethoxazole target the highest specificity and albeit with correspondingly lower sensitivity (Tables 2 and 5).

Observed agreement estimates were strong for most antimicrobials on resistant breakpoints (range, 0.94–1.0), but highly variable using susceptible breakpoints (range, 0.39–0.99) (Table 4). (Supplementary Tables 3 and 4 outline the contribution of positive agreement and negative agreement towards overall observed agreement estimates using susceptible and resistant breakpoints). Antimicrobials with greater than 1% difference between proportion resistant by broth-microdilution and proportion resistant by disc diffusion recorded a statistically significant ($p < 0.05$) mid-p value McNemar's test (Table 4). Amoxicillin-clavulanic acid, cephalothin and cefoxitin recorded excessively large differences between the proportions resistant by broth-microdilution and disc diffusion based on susceptible breakpoints. These three antimicrobials also performed sub-optimally when inter-test agreement was measured by PABAK (Table 4). Antimicrobials with the lowest disc diffusion performance estimates also had increased overlapping susceptible and non-susceptible populations (Fig. 3). Disc diffusion estimates of

Table 4
Agreement estimates between broth-microdilution and disc diffusion for 994 clinical *Escherichia coli* isolates from animals using CLSI susceptible and resistant breakpoints. Exact 95% confidence intervals for estimates are in Supplementary materials. BMD, broth-microdilution; DD, disc diffusion.

	Susceptible breakpoint estimates					Resistant breakpoint estimates				
Antimicrobial	BMD resistant	DD resistant	McNemars p-value	Observed agreement	PABAK	BMD resistant	DD resistant	McNemars p-value	Observed agreement	PABAK
Amoxicillin-clavulanic acid	0.79	0.18	< 0.001*	0.39	NA	0.10	0.09	< 0.001*	0.97	0.95
Amikacin	0.02	0.01	0.02*	0.97	0.94	0.02	0.02	0.63	1.0	NA
Ampicillin	0.35	0.45	< 0.001*	0.85	0.70	0.28	0.30	< 0.001*	0.96	0.92
Cephalothin	0.92	0.66	< 0.001*	0.71	0.41	0.20	0.17	< 0.001*	0.94	0.87
Ceftiofur	0.11	0.11	0.20	0.97	0.94	0.10	0.10	0.77	0.99	0.98
Ciprofloxacin	0.08	0.08	0.73	0.99	0.99	0.07	0.07	0.63	1.0	0.99
Cefovecin	0.15	0.14	0.08	0.92	0.84	0.10	0.10	0.17	0.98	0.97
Cefoxitin	0.25	0.09	< 0.001*	0.83	0.65	0.09	0.08	0.05*	0.98	0.96
Gentamicin	0.10	0.06	< 0.001*	0.94	0.89	0.05	0.05	0.73	0.99	0.99
Imipenem	0.04	0.02	< 0.001*	0.95	0.89	0	0	0.2	1.0	0.99
Trimethoprim-sulfamethoxazole	0.21	0.15	< 0.001*	0.93	0.86	0.19	0.15	< 0.001*	0.94	0.88
Tetracycline	0.19	0.19	0.85	0.97	0.95	0.18	0.18	0.30	0.99	0.97

NA, not available due to insufficient data for analysis.
* Significant mid-*p* McNemar's chi-square test ($p < 0.05$).

Table 5
Estimates of accuracy of disc diffusion relative to broth-microdilution for 994 clinical *Escherichia coli* isolates from animals using zone diameter interpretative criteria produced from the dBETS program. DSe, diagnostic sensitivity; DSp diagnostic specificity; ZD, zone diameter. Exact 95% confidence intervals for estimates provided in Supplementary materials.

	dBETS Susceptible Breakpoint Estimates				dBETS Resistant Breakpoint Estimates			
Antimicrobial	ZD susceptible breakpoint (mm)	Relative DSe	Relative DSp	Observed agreement	ZD resistant breakpoint (mm)	Relative DSe	Relative DSp	Observed agreement
Amoxicillin-clavulanic acid	21	0.70	0.87	0.66	15	0.92	0.98	0.98
Amikacin	16	NA	1.0	0.97	12	NA	1.0	1.0
Ampicillin	11	0.80	0.98	0.92	7	0.96	0.98	0.97
Cephalothin	18	0.70	0.81	0.71	13	0.68	0.99	0.93
Ceftiofur	22	0.86	0.98	0.97	18	0.96	0.99	0.99
Ciprofloxacin	18	0.96	1.0	1.0	11	0.90	1.0	0.99
Cefovecin	23	0.67	0.96	0.92	19	0.88	0.99	0.98
Cefoxitin	22	0.43	0.97	0.83	18	0.91	0.99	0.98
Gentamicin	16	0.50	0.99	0.94	12	0.92	1.0	0.99
Imipenem	23	NA	0.99	0.95	15	NA	1.0	1.0
Trimethoprim-sulfamethoxazole	25	0.87	0.86	0.86	21	0.79	0.98	0.94
Tetracycline	18	0.93	0.99	0.97	13	0.95	0.99	0.98

NA, not available due to insufficient data for the analysis.

accuracy are optimised when there is clear separation of 'susceptible' and 'non-susceptible' populations as demonstrated on the zone diameter histograms for ciprofloxacin, tetracycline, and ceftiofur (Fig. 3). However, disc diffusion estimates are weaker when susceptible and non-susceptible populations overlap (e.g. amoxicillin-clavulanic acid, cephalothin, and cefoxitin).

Improved disc diffusion performance estimates were produced when dBETS zone diameter susceptible breakpoints were applied (Table 5). This was particularly evident for amoxicillin-clavulanic acid where sensitivity went from 0.23 using the CLSI susceptible breakpoint to 0.61. However cefoxitin (CLSI: 0.33; dBETS 0.43) and gentamicin (CLSI: 0.50, dBETS: 0.50) estimates were minimally improved. At the resistant breakpoint, disc diffusion performance was relatively unchanged when the dBETS values were applied. At dBETS susceptible breakpoint, observed agreement for many of the antimicrobials evaluated was improved (Table 5) compared to CLSI susceptible breakpoints (Table 4).

4. Discussion

Inferences made in this work are based on a large number of clinical *E. coli* isolates (n = 994) from multiple animal species, and procured from a formal survey involving all major veterinary laboratories in Australia. The most notable finding of this study is the marked superiority in the performance of disc diffusion relative to broth-microdilution when assessed on resistant breakpoints compared to susceptible breakpoints. When resistant breakpoints are applied to broth-microdilution results, a very high level of disc diffusion relative accuracy is evident for the majority of antimicrobials evaluated, particularly for critically important antimicrobials (i.e. fluoroquinolones and third-generation cephalosporins). In comparison, disc diffusion performance was lower for most antimicrobials at susceptible breakpoints. This study also provides dBETS zone diameter breakpoints which have a greater objective basis than the current approach used to establish CLSI zone diameter breakpoints. The performance of disc diffusion for amoxicillin-clavulanic acid, cefoxitin and gentamicin was particularly sensitive to the choice of breakpoints, resulting in highly variable sensitivity estimates and large discrepancies in observed agreement. Cephalothin and trimethoprim-sulfamethoxazole had poor disc diffusion performance estimates regardless of the breakpoint used to dichotomise the data.

Observations arising from this study demonstrate that disc diffusion is appropriate to differentiate a population of clinical *E. coli* isolates derived from animals using CLSI or dBETS zone diameter *resistant* breakpoints for the majority of antimicrobials assessed in this study. However, for several antimicrobials including amoxicillin-clavulanic

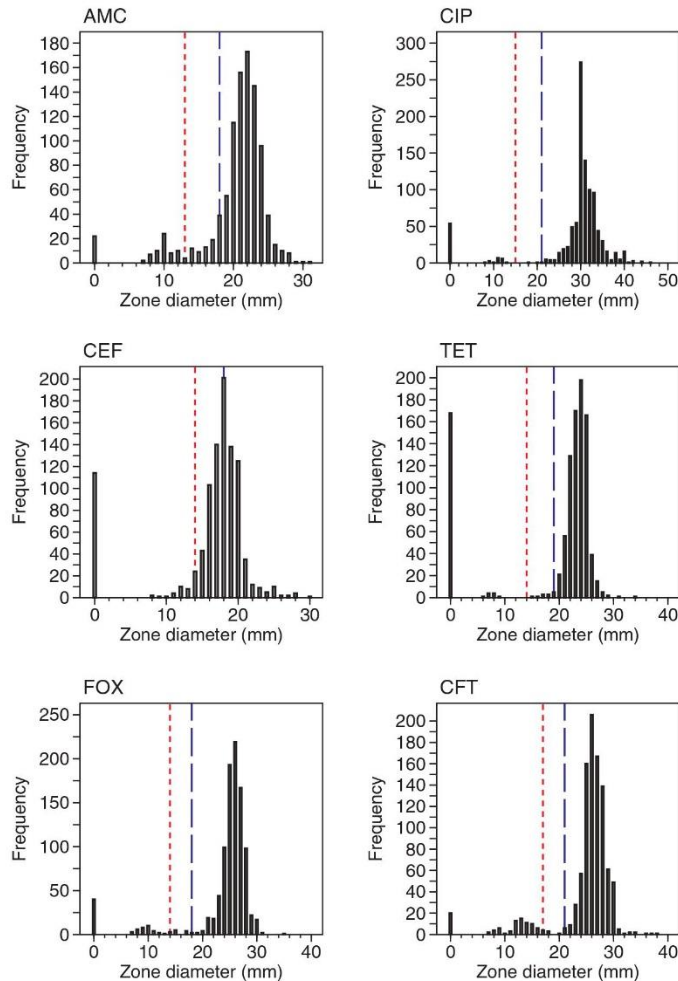

Fig. 3. Distribution of zone diameter results for clinical *E. coli* isolates derived from animals (n = 994) for six antimicrobials. CLSI resistant breakpoint (red short-dash line) and susceptible breakpoint (blue long-dash line) is plotted over each distribution. AMC, amoxicillin-clavulanic acid; CIP, ciprofloxacin; CEF, cephalothin; TET, tetracycline; FOX, cefoxitin; CFT, ceftiofur. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

acid, cefoxitin and gentamicin, disc diffusion has limitations when differentiating a population of clinical *E. coli* isolates using CLSI zone diameter *susceptible* breakpoints. Susceptible zone diameter breakpoints generated by dBETS are sometimes superior and should be considered when breakpoints are established. These findings also inform on the selection of antimicrobials for inclusion in national surveillance, with disc diffusion estimates for ciprofloxacin ceftiofur, ampicillin and tetracycline proving robust across breakpoints.

The study outcomes also support improved clinical decision-making by providing robust estimates of sensitivity and specificity for disc diffusion that hitherto have been rarely reported. These parameters, along with likelihood ratio pairs and ROC analysis, are key metrics relied upon in evidence-based approaches to clinical decision-making and the assessment of diagnostic test performance (Dohoo et al., 2009; OIE, 2017a). Moreover in a surveillance setting, the 'true' prevalence (Rogan and Gladen, 1978) of resistance in a population can be estimated if sensitivity and specificity are known. Calculating true prevalence from sensitivity and specificity will adjust for the inaccuracy of disc diffusion (i.e. apparent prevalence) and allow for comparison of zone diameter prevalence with MIC prevalence. This will improve the validity of surveillance data obtained from clinical *E. coli* isolates from animals. Thus, the quantitative estimates of test performance provided here for a broad panel of antimicrobials stands to benefit both population health and clinical medicine.

ROC analysis is useful to determine test accuracy and assist in defining breakpoint values however, only a small number of microbiology studies have utilised ROC analysis for determination of performance of phenotypic susceptibility assays in veterinary isolates (Jean et al., 2015; Klement et al., 2005; Saini et al., 2011; Schumacher et al., 2001). Hanczar et al. (2010) identified the need for large sample sizes in ROC estimation of assay performance (Hanczar et al., 2010) which has been achieved in this study. Although efforts have been made to utilise ROC analysis for veterinary pathogens, the sample size in such studies has been small, for example Saini et al. (2011) perform ROC analysis for disc diffusion using a sample of 25 *E. coli* isolates, and Klement et al. (2005) used 231 *E. coli* isolates from bovine milk samples.

Discrepancies in disc diffusion performance estimates for some antimicrobials found here are in agreement with other studies (Hombach et al., 2013; Klement et al., 2005). While variable performance estimates may be attributed to biological differences, technical limitations (including laboratory error), or true variation in the disc diffusion test, the appropriateness of the breakpoints must also be considered. Not all antimicrobials evaluated in this study have breakpoints specific for veterinary isolates, making it necessary to use human breakpoints. This

has likely resulted in variable disc diffusion performance estimates for some drugs. Additionally for trimethoprim-sulfamethoxazole, the trailing endpoint phenomenon seen with MIC assays (Jorgenson and Turnidge, 2015) may be responsible for variability in disc diffusion performance results. Epidemiological cut off points (ECOFFs) are often used as the basis for performing surveillance (Silley, 2012). However, owing to the existing complexity of this study (involving 12 antimicrobials and use of two breakpoints) ECOFFs were not included in the analysis. Nevertheless, ECOFFs for a given drug are often similar to, or lower than CLSI susceptible breakpoints and the conclusion of reduced test accuracy for disc diffusion compared to broth microdilution will also hold for interpretations based on ECOFFs. It was also evident in this study that overlapping susceptible and non-susceptible populations resulted in misclassification errors. In this study, misclassification errors were retained to replicate the imperfections that would likely occur if the veterinary laboratory network were to submit routine disc diffusion data for use in national surveillance. The dBETS method appeared relatively robust to outliers for most of the antimicrobials assessed.

Limitations associated with this study should be considered. This study only examined clinical *E. coli* isolates therefore the findings should not be generalized to non-pathogenic (commensal) *E. coli* from healthy animals typically included in AMR surveillance. Data for this study was generated in a single laboratory and does not accommodate the possibility of laboratory-to-laboratory variation (reproducibility) in test performance. Broth-microdilution is an imperfect reference test and the performance estimates for disc diffusion can never exceed those of broth-microdilution. Theoretically, better disc diffusion accuracy estimates can be obtained by latent class analysis (Pepe and Janes, 2007) which is not reliant on a perfect reference test, however the assumptions that underlie this approach precludes its use in this study. While accuracy measures such as sensitivity, specificity, and AUC provide the best available evidence of inter-test compatibility, agreement measures such as observed agreement, McNemars test, and inter-test agreement have been reported in this study to facilitate comparison with previous studies. In the future, the existing isolate collection will be expanded to aid in the development of clinical breakpoints unique for animal species, disease syndromes or combinations of these.

5. Conclusion

We have demonstrated that for most antimicrobials, disc diffusion was shown to be accurate at predicting the resistance status of animal-derived clinical *E. coli* that could otherwise be obtained by broth-microdilution. However, for a sub-set of antimicrobials disc diffusion demonstrated inferior performance relative to broth-microdilution and this warrants further investigation. Although disc diffusion performance at the susceptible breakpoint is suboptimal, standard equations can be applied to correct this. Moreover, these findings inform on the selection of antimicrobials for inclusion in national surveillance, with disc diffusion performing well for critically important antimicrobial classes such as fluoroquinolones and third-generation cephalosporins. For these reasons disc diffusion appears to have applicability in national surveillance provided performance of the assay, as defined in this work, is taken into account.

Ethical approval

None required.

Declaration of interest statement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetmic.2017.12.008.

References

Abraham, S., Jordan, D., Wong, H.S., Johnson, J.R., Toleman, M.A., Wakeham, D.L., Gordon, D.M., Turnidge, J.D., Mollinger, J.L., Gibson, J.S., Trott, D.J., 2015. First detection of extended-spectrum cephalosporin- and fluoroquinolone-resistant Escherichia coli in Australian food-producing animals. Journal of Global Antimicrobial Resistance 3, 273–277.

Benedict, K.M., Gow, S.P., Checkley, S., Booker, C.W., McAllister, T.A., Morley, P.S., 2013. Methodological comparisons for antimicrobial resistance surveillance in feedlot cattle. BMC Vet. Res. 9, 216.

Byrt, T., Bishop, J., Carlin, J.B., 1993. Bias, prevalence and kappa. J. Clin. Epidemiol. 46, 423–429.

CLSI, 2013. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals (VET01-A4), 4th edition. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI, 2015a. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals (VET01-S3), 3rd edition. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI, 2015b. Performance standards for antimicrobial susceptibility testing; (M100-S25). 25th Informational Supplement. Clinical and Laboratory Standards Institute, Wayne, PA.

DePalma, G., Turnidge, J., Craig, B.A., 2017. Determination of disk diffusion susceptibility testing interpretive criteria using model-based analysis: development and implementation. Diagn. Microbiol. Infect. Dis. 87, 143–149.

Dohoo, I., Martin, W., Stryhn, H., 2009. Screening and diagnostic tests. Veterinary Epidemiologic Research. VER Inc, Charottetown, pp. 91–127.

Fagerland, M.W., Lydersen, S., Laake, P., 2013. The McNemar test for binary matched-pairs data: mid-p and asymptotic are better than exact conditional. BMC Med. Res. Methodol. 13, 91.

Greiner, M., Gardner, I.A., 2000. Epidemiologic issues in the validation of veterinary diagnostic tests. Prev. Vet. Med. 45, 3–22.

Hanczar, B., Hua, J., Sima, C., Weinstein, J., Bittner, M., Dougherty, E.R., 2010. Small-sample precision of ROC-related estimates. Bioinformatics 26, 822–830.

Hoelzer, K., Cummings, K.J., Warnick, L.D., Schukken, Y.H., Siler, J.D., Groehn, Y.T., Davis, M.A., Besser, T.E., Wiedmann, M., 2011. Agar disk diffusion and automated microbroth dilution produce similar antimicrobial susceptibility testing results for Salmonella serotypes newport, typhimurium, and 4,5,12:i-, but differ in economic cost. Foodborne Pathog. Dis. 8, 1281–1288.

Hombach, M., Bottger, E.C., Roos, M., 2013. The critical influence of the intermediate category on interpretation errors in revised EUCAST and CLSI antimicrobial susceptibility testing guidelines. Clin. Microbiol. Infect. 19, E59–E71.

ISO, 2006. Clinical laboratory testing and in vitro diagnostic test systems – susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices. Part 1: Reference Method for Testing the in Vitro Activity of Antimicrobial Agents Against Rapidly Growing Aerobic Bacteria Involved in Infectious Diseases. 20776-1:2006. International Organisation for Standardisation.

Jean, S.S., Liao, C.H., Sheng, W.H., Lee, W.S., Hsueh, P.R., 2015. Comparison of commonly used antimicrobial susceptibility testing methods for evaluating susceptibilities of clinical isolates of Enterobacteriaceae and nonfermentative Gram-negative bacilli to cefoperazone-sulbactam. J. Microbiol. Immunol. Infect.

Jorgenson, J.H., Turnidge, J., 2015. Susceptibility test methods: dilution and disk diffusion methods. In: Jorgensen, J.H., Pfaller, Michael A. (Eds.), Manual of Clinical Microbiology. ASM Press, Washington, pp. 1253–1273.

Klement, E., Chaffer, M., Leitner, G., Shwimmer, A., Friedman, S., Saran, A., Shpigel, N., 2005. Assessment of accuracy of disk diffusion tests for the determination of antimicrobial susceptibility of common bovine mastitis pathogens: a novel approach. Microb. Drug Resist.-Mech. Epidemiol. Dis. 11, 342–350.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A.K., Wertheim, H.F., Sumpradit, N., Vlieghe, E., Hara, G.L., Gould, I.M., Goossens, H., Greko, C., So, A.D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A.Q., Qamar, F.N., Mir, F., Kariuki, S., Bhutta, Z.A., Coates, A., Bergstrom, R., Wright, G.D., Brown, E.D., Cars, O., 2013. Antibiotic resistance-the need for global solutions. Lancet Infect. Dis. 13, 1057–1098.

OIE, 2015. Resolution 26. Combating antimicrobial resistance and promoting the prudent

use of anitmicrobial agents in animals. In: 83rd General Assembly. Paris. . http://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/AMR/A_RESO_AMR_2015. pdf.

OIE, 2017a. Chapter 1.1.6. Principles and methods of validation of diagnostic assays for infectious diseases. Adopted 2013. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 7th edition. . http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.06_VALIDATION.pdf.

OIE, 2017b. Chapter 3.1 laboratory methodologies for bacterial antimicrobial susceptibility testing. Adopted 2012. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 7th edition. . http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.1_ANTIMICROBIAL.pdf.

Pepe, M.S., Janes, H., 2007. Insights into latent class analysis of diagnostic test performance. Biostatistics 8, 474–484.

Rhodes, N.J., Richardson, C.L., Heraty, R., Liu, J., Malczynski, M., Qi, C., Scheetz, M.H., 2014. Unacceptably high error rates in Vitek 2 testing of cefepime susceptibility in extended-spectrum-beta-lactamase-producing Escherichia coli. Antimicrob. Agents Chemother. 58, 3757–3761.

Rogan, W.J., Gladen, B., 1978. Estimating prevalence from the results of a screening test. Am. J. Epidemiol. 107, 71–76.

Saini, V., Riekerink, R.G., McClure, J.T., Barkema, H.W., 2011. Diagnostic accuracy assessment of Sensititre and agar disk diffusion for determining antimicrobial resistance profiles of bovine clinical mastitis pathogens. J. Clin. Microbiol. 49, 1568–1577.

Schumacher, H., Hoffmann, S., Holmboe, C., Moller, J.K., 2001. A procedure for evaluation and documentation of susceptibility test methods using the susceptibility of Klebsiella pneumoniae to ciprofloxacin as a model. J. Antimicrob. Chemother. 48, 493–500.

Shaban, R., Simon, G., Trott, D., Turnidge, J., Jordan, D., 2014. Surveillance and Reporting of Antimicrobial Resistance and Antibitoic Usage in Animals and Agriculture in Australia (Canberra).

Silley, P., 2012. Susceptibility testing methods, resistance and breakpoints: what do these terms really mean? Rev. Sci. Et Tech.-Off. Int. Des Epizoot. 31, 33–41.

Turnidge, J., Paterson, D.L., 2007. Setting and revising antibacterial susceptibility breakpoints. Clin. Microbiol. Rev. 20, 391–408.

United Nations 2016. At UN, global leaders commit to act on antimicrobial resistance. Collective effort to address a challenge to health, food security and development. Joint News Release, 21 September 2016, OPGA/WHO/FAO/OIE, ed. (New York, http://www.who.int/mediacentre/news/releases/2016/commitment-antimicrobial-resistance/en/).

WHO, 2015a. AGISAR 5-Year strategic framework to support implementation of the global action plan on antimicrobial resistance 2015–2019. In: Report of the 6th Meeting. Advisory Group on Integrated Surveillance of Antimicrobial Resistance. Seoul. . http://apps.who.int/iris/bitstream/10665/190954/1/9789241509534_eng.pdf?ua=1.

WHO, 2015b. Global action plan on antimicrobial resistance. In: 68th World Health Assembly. Geneva. . http://www.wpro.who.int/entity/drug_resistance/resources/global_action_plan_eng.pdf.

Supplementary materials for Chapter 2 can be found in Appendix 1

Chapter 3:

Diagnostic accuracy of phenotypic assays for determining antimicrobial resistance status in *Staphylococcus pseudintermedius* isolates from canine clinical cases

Contextual Statement

The preceding chapter detailed the accuracy of disc diffusion relative to broth microdilution for clinical *Escherichia coli* isolates derived from animals. In this chapter, the accuracy of disc diffusion relative to broth microdilution is evaluated against *Staphylococcus. pseudintermedius*, an important and ubiquitous bacterium of dogs. Chapter 3 builds on Chapter 2 by evaluating the performance of both disc diffusion and broth microdilution to a more accurate test – *mecA* real-time PCR for the prediction of methicillin resistance. Few studies have assessed the performance attributes of broth microdilution even though it is widely considered to be the reference test to which all other phenotypic assays are compared. As genetic and molecular technologies become accessible, opportunities exist to evaluate the performance of broth microdilution fully. In this study, paired zone diameter and minimum inhibitory concentration (MIC) measurements from 614 clinical *S. pseudintermedius* isolates were used in analyses, with isolates also tested by real-time PCR. Conventional statistical methods were used to evaluate the accuracy of disc diffusion, including the reporting of diagnostic sensitivity and specificity and use of receiver-operating characteristic (ROC) analysis.

Statement of Authorship

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Contribution to the Paper	Study design, performed analysis and interpreted data, wrote manuscript, and acted as corresponding author for liaison with editor.		
Overall percentage (%)	75		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Diagnostic accuracy of phenotypic assays for determining antimicrobial resistance status in *Staphylococcus pseudintermedius* isolates from canine clinical cases

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ABSTRACT

This study evaluated the diagnostic test accuracy of disc diffusion relative to broth-microdilution for clinical *Staphylococcus pseudintermedius* isolated from dogs in Australia (n = 614). Accuracy of disc diffusion and broth-microdilution for oxacillin relative to *mecA* real-time PCR was also assessed. Each isolate had paired minimum inhibitory concentration and zone diameter values for ten antimicrobial agents. Data was dichotomised using Clinical and Laboratory Standards Institute susceptible and resistant clinical breakpoints. Test accuracy was reported using relative diagnostic sensitivity (RSe), specificity (RSp), likelihood ratio pairs, diagnostic odds ratio, and area-under-the receiver-operating characteristic (ROC AUC) analysis. Disc diffusion was found to have high test accuracy for most antimicrobials (ROC AUC range: 0.96 – 0.99) except rifampicin (ROC AUC = 0.80). The RSp of disc diffusion was high for all antimicrobials (range, 97.1%–100%). However, RSe was considerably variable (range, 35.7%–98.8%), particularly for amoxicillin-clavulanic acid (51.5%, 95% CI, 38.9%, 64.0%), cefoxitin (35.7%, 95% CI, 12.8%, 64.9%), and cephalothin (43.6%, 95% CI, 27.8%, 60.4%). When disc diffusion and broth-microdilution were compared to *mecA* real-time PCR, the overall accuracy of both assays was similar (ROC AUC, 0.99 respectively). However, the RSe for broth-microdilution (96.1%, 95% CI, 88.9%, 99.2%) was significantly higher than for disc diffusion (86.8%, 95% CI, 77.1%, 93.5%) (McNemars mid-*p* value 0.01). Overall, these findings demonstrate that for most antimicrobials, disc diffusion performed according to CLSI guidelines can be used to differentiate clinical *S. pseudintermedius* isolates that might otherwise be assessed by broth-microdilution, provided consideration is given to the performance estimates reported here.

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1. Introduction

The acquisition of resistance genes in clinically important bacterial pathogens of animals is of great concern. In particular, methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) poses a major challenge owing to extensive multidrug resistance, limited therapeutic options for infected hosts, and in rare cases, risk of sporadic zoonotic infection in people (van Duijkeren et al., 2011). For these reasons, accurate assessment of *S. pseudintermedius* susceptibility to a wide range of antimicrobials is required, not only as an objective basis for clinical therapy but also to monitor the occurrence of antimicrobial resistance in animal populations.

In most staphylococci, methicillin resistance is mediated by the mecA gene which encodes expression of the modified penicillin-binding protein, PBP2a (CLSI, 2015). Clinical and Laboratory Standards Institute (CLSI) guidelines specify the most reliable test for determination of methicillin resistance in *S. pseudintermedius* is detection of *mecA* by polymerase chain reaction (PCR) (CLSI, 2018). However, few veterinary laboratories routinely perform *mecA* PCR due to cost to owners. Typically, diffusion- or dilution-based phenotypic assays (using oxacillin as a surrogate for methicillin) are used to predict methicillin resistance, or more accurately, pan-beta-lactam resistance (CLSI, 2018). In most veterinary laboratories, diffusion-based assays such as disc diffusion are performed rather than dilution-based assays such as broth-microdilution (Hardefeldt et al., 2018). However, for most national surveillance programs, broth-microdilution is the preferred assay to generate antimicrobial susceptibility data in animals.

Comprehensive surveillance of antimicrobial resistance in animal pathogens such as *S. pseudintermedius* may be achieved if susceptibility data generated from disc diffusion can be acquired from those veterinary laboratories that routinely evaluate such pathogens. However, the acquisition of susceptibility data is only of value if the accuracy of disc diffusion is comparable to that of broth-microdilution. Additionally, factors associated with assay performance including consistent application of standardised protocols for identification of isolates, quality control, conduct of the test, antibiotic panels, and reading of zones must be widely adopted.

At present, there is limited understanding of the accuracy of disc diffusion when it is compared to broth-microdilution, especially in relation to animal pathogens. This poses a barrier to the use of disc diffusion susceptibility data in national surveillance programs. Standard statistical measures, such as relative diagnostic sensitivity and specificity, receiver-operating characteristic (ROC) analysis, area-under-the-curve (AUC), likelihood ratios, and diagnostic odds ratios (DOR) for a range of antimicrobial agents are essential to determine if disc diffusion and broth-microdilution generate comparable results. However, few studies have reported these estimates for disc diffusion in relation to *S. pseudintermedius*. Indeed, Bremis et al (2006, 2009) and Schissler et al (2009) are the only studies where diagnostic sensitivity and specificity of disc diffusion were reported although these studies limited analysis to oxacillin and cefoxitin alone. Other studies have also evaluated the appropriateness of oxacillin or cefoxitin interpretative criteria to predict methicillin resistance compared to the presence of the *mecA* gene (Bemis et al., 2012; Schmidt et al., 2014; Siak et al., 2014; Wu et al., 2016). However, analysis in these studies was mostly restricted to descriptive measures such as categorical agreement and error-rates, estimates which cannot be relied upon alone to adequately describe the accuracy of disc diffusion.

Therefore, the objective of this study was to evaluate the relative accuracy of disc diffusion compared to broth-microdilution to a range of antimicrobials for clinical *S. pseudintermedius* isolates derived from dogs. In addition, the accuracy of disc diffusion and broth-microdilution was compared to *mecA* real-time PCR for the prediction of methicillin resistance. An improved understanding of the accuracy of these assays will help determine if susceptibility data from *S. pseudintermedius* can be included in national surveillance.

2. Methods

2.1. Sample acquisition, characterisation, and antimicrobial susceptibility testing

S. pseudintermedius isolates were derived from a structured survey of antimicrobial resistance in veterinary pathogens between January 2013 and January 2014, involving all 22 veterinary diagnostic laboratories in Australia (Saputra et al., 2017). Coagulase-positive staphylococci isolates considered clinically relevant to the presenting condition (as determined by the diagnostic microbiologist) were sent to The University of Adelaide during the collection period. Species identification was confirmed by the University of Adelaide reference laboratory using the BD™ Bruker MALDI Biotyper. Isolates in the collection underwent broth-microdilution (minimum inhibitory concentration, MIC, μg/ml) and disc diffusion (zone diameter, mm) testing according to CLSI VET01-A4 protocols (CLSI, 2013b) at the University of Adelaide reference laboratory. *Staphylococcus aureus* ATCC 25923 and ATCC 29213 were used as quality control strains. The MIC results for the isolates were obtained from a previous study (Saputra et al., 2017). Disc diffusion testing was performed independently to when broth-microdilution testing occurred. Antibiotic discs were obtained from Thermo-Fischer Scientific (Australia). Antibiotics evaluated in this study are listed in Table 1. An antibiotic was included for evaluation if there were corresponding MIC and zone diameter measurements for isolates included in the study.

Table 1
Disc diffusion and broth-microdilution Clinical Laboratory Standards Institute (CLSI) interpretative criteria for canine clinical *Staphylococcus pseudintermedius* isolates (n = 614) evaluated in this study.

			Broth-microdilution (μg/ml)			Disc Diffusion (mm)	
Antimicrobial	Abbreviation	Disc content (ug)	MIC range	Susceptible breakpoint	Resistant breakpoint	Susceptible breakpoint	Resistant breakpoint
Amoxicillin-clavulanic acid[a]	AMC	20/10	0.06 – 32	≤4/2	≥8/4	≥20	≤19
Cefovecin[b]	CVN	30	0.06 – 64	≤0.5	≥2	≥24	≤20
Cefoxitin[b]	FOX	30	0.06 – 64	≤4	≥8	≥22	≤21
Cephalothin[a]	CEF	30	0.06 – 64	≤8	≥32	≥18	≤14
Chloramphenicol[b]	CHL	30	2.0 – 64	≤8	≥32	≥18	≤12
Ciprofloxacin[c]	CIP	5	0.03 – 8	≤1	≥4	≥21	≤15
Clindamycin[b]	CLI	2	0.03 – 32	≤0.5	≥4	≥21	≤14
Oxacillin[b]	OXA	1	0.03 – 64	≤0.25	≥0.5	≥18	≤17
Rifampicin[b]	RIF	5	0.004 – 4	≤1	≥4	≥20	≤16
Tetracycline[b]	TET	30	0.06 – 64	≤0.25	≥1	≥23	≤17

[a] CLSI VET01S2: Table 2B. Human Derived Zone Diameter Interpretation Standards and Minimum Inhibitory Concentration Breakpoints for Veterinary Pathogens.
[b] CLSI VET08: Table 2C. Zone Diameter and Minimum Inhibitory Concentration Breakpoints for *Staphylococcus* spp.
[c] CLSI M100-S25. Table 2C. Zone Diameter Interpretative Standards and MIC Breakpoints for *Staphylococcus* spp.

2.2. Screening for mecA

To assess *mecA* status, copies of the collection were sent to the Antimicrobial Resistance and Infectious Diseases Laboratory at Murdoch University. Isolates were cultured from frozen (−80 °C) stock culture according to CLSI protocols (CLSI, 2013b). DNA was extracted as described in Abraham et al. (2012) with minor modifications (Abraham et al., 2018). Presence of the *mecA* gene was determined by singleplex real-time probe-based PCR, as described previously (Costa et al., 2005). The probe (FAM-TTCCAGGAATGCAGAAAGACCAAAGCA-BHQ) and forward primer (5`-TGGTATGTGGAAGTTAGATTGGGAT-3`) used in this study were identified in a previous study (Nakagawa et al., 2005). The reverse primer was identified by performing a Basic Local Alignment Search Tool (BLAST) search on the online NCBI GenBank database. Based on sequence alignment, the reverse primer was designed manually (5`-CTATCTCATATGCTGTTCCTGTATTGGC-3`). MRSP isolates previously characterised by whole-genome sequencing for strain typing and detection of *mecA* genes by Worthing et al (2018a) were used as controls for comparison. Real-time PCR was performed in duplicate in 96-well plates using a 10 μL reaction mixture on QuantStudio™ 6 Flex Real-Time PCR system (Thermo Fisher Scientific, Australia). The reaction mixture comprised a final concentration of 0.4μM of each primer, 0.2μM probe, 5 μL TaqMan® Fast Advanced Master Mix, 2 μL nuclease-free water, and 2 μL DNA template.

3. Whole genome sequencing

Isolates with discordant oxacillin phenotypic and *mecA* real-time PCR results underwent whole genome-sequencing. DNA extractions were performed using a MagMax DNA multi-sample kit (ThermoFisher Scientific) according to the manufacturer's instructions, with the modification to omit the RNAse treatment step. Library preparation was performed with a Nextera XT kit with an increased tagmentation time of seven minutes. Sequencing was performed on an Illumina Nextseq 500 platform using a high-output V2 (2 × 150 cycles) reagent kit (O'Dea et al., 2018). Sequencing files were uploaded to the Center for Genomic Epidemiology (https://genomicepidemiology.org) and the ResFinder application used to check for the presence of acquired resistance genes.

3.1. Data analysis

Data used in this study comprised paired MIC and zone diameter values for 614 canine clinical *S. pseudintermedius* isolates for ten antibiotics.

For evaluation of test accuracy, MIC and zone diameter values were dichotomised using corresponding CLSI clinical interpretative criteria (Table 1). Where veterinary-specific *S. pseudintermedius* clinical breakpoints were unavailable, veterinary-specific *Staphylococcus* genus clinical breakpoints were used. Where veterinary-specific clinical breakpoints were unavailable or did not have corresponding MIC and zone diameter breakpoints, human breakpoints were used. For amoxicillin-clavulanic acid, we used the corresponding zone diameter and MIC breakpoints given by the last CLSI document containing them – CLSI VET01-S2, Table 2B (2013a). When the susceptible breakpoint was used to dichotomise MIC and zone diameter results, isolates in the 'intermediate' and 'resistant' range were collectively classified as 'non-susceptible'. When data were dichotomised using the resistant clinical breakpoint, isolates were classified as 'non-resistant' if their MIC or zone diameter value was within the 'intermediate' or 'susceptible' range. For real-time PCR, samples showing a sigmoidal curve with a cycle threshold (C_T) ≤ 40 were considered positive for the presence of *mecA* and negative if no sigmoidal curve was observed.

The accuracy of disc diffusion classification relative to broth-microdilution (the reference method) was evaluated by estimating relative diagnostic sensitivity (RSe) and specificity (RSp), likelihood ratios of positive (LR+) and negative (LR-) results and summarised using DOR and ROC AUC. For evaluation of the accuracy of disc diffusion and broth-microdilution to predict methicillin resistance in *S. pseudintermedius*, *mecA* real-time PCR was the reference test. ROC plots and AUC were estimated using non-parametric analysis since MIC data cannot be assumed to be normally distributed. Two-graph (TG) ROC plots were used to visualise the relative accuracy of disc diffusion to broth-microdilution across a range of cut-off values. Details on diagnostic sensitivity, specificity, LR pairs, DOR, and ROC analysis are given elsewhere (Glas et al., 2003; Greiner and Gardner, 2000).

Observed agreement was calculated as the proportion of isolates with the same interpretative classification by disc diffusion and broth-microdilution. Similarly, agreement between broth-microdilution, disc diffusion, and real-time PCR was calculated as the proportion of isolates with the same oxacillin interpretative classification and *mecA* status. McNemar's mid-*p* test was used to assess the extent of disagreement between two tests where a P value < 0.05 was considered significant (Fagerland et al., 2013).

Data were entered into MS Excel files and imported into Stata version 15.1 (Stata Corporation, College Station, TX) for all analysis. Data is available in supplementary information.

4. Results

4.1. Disc diffusion accuracy relative to broth-microdilution

RSp was consistently high across all antimicrobials (range, 97.1%–100%), whereas RSe estimates showed considerable variability (range, 35.7%–98.8%) when zone diameter and MIC values were dichotomised using susceptible breakpoints (Table 2). Similar results were recorded when resistant breakpoints were applied. Poor RSe estimates were recorded for amoxicillin-clavulanic acid (51.5%, 95% CI,

Table 2
Diagnostic performance estimates of disc diffusion relative to broth-microdilution for 614 canine *Staphylococcus pseudintermedius* isolates from clinical cases. Clinical and Laboratory Standards Institute (CLSI) susceptible and resistant breakpoints were used to dichotomise minimum inhibitory concentration (MIC) and zone diameter values. RSe, relative diagnostic sensitivity; RSp, relative diagnostic specificity; AUC, area-under-the-curve. Exact 95% confidence intervals are given.

	Susceptible Breakpoint Estimates			Resistant Breakpoint Estimates		
Antimicrobial	%RSe (95%CI)	%RSp (95%CI)	AUC (95%CI)	%RSe (95%CI)	%RSp (95%CI)	AUC
Amoxicillin-clavulanic acid	51.5 (38.9, 64.0)	99.8 (99.0, 100.0)	0.99 (0.97, 1.0)	51.5 (38.9, 64.0)	99.8 (90.0, 100.0)	0.99 (0.97, 1.0)
Cefovecin	72.3 (61.4, 81.6)	99.1 (97.8, 99.7)	0.95 (0.92, 0.98)	85.7 (74.6, 93.3)	98.6 (97.2, 99.4)	0.99 (0.98, 1.0)
Cefoxitin	35.7 (12.8, 64.9)	99.3 (98.3, 99.8)	0.96 (0.92, 1.0)	35.7 (12.8, 64.9)	99.3 (98.3, 99.8)	0.96 (0.92, 1.0)
Cephalothin	66.7 (51.1,80.0)	99.7 (98.7, 99.7)	0.99 (0.97, 1.0)	43.6 (27.8, 60.4)	99.8 (99.0, 100.0)	0.98 (0.97, 1.0)
Chloramphenicol	85.0 (70.2, 94.3)	100.0 (99.4, 100.0)	0.97 (0.93, 1.0)	94.4 (81.3, 99.3)	100.0 (99.4, 100.0)	0.99 (0.98, 1.0)
Ciprofloxacin	90.9 (80.1, 97.0)	99.8 (99.0, 100.0)	0.98 (0.96, 1.0)	88.2 (76.1, 95.6)	99.8 (99.0, 100.0)	0.98 (0.96, 1.0)
Clindamycin	98.8 (93.4, 100.0)	99.3 (98.1, 99.8)	0.99 (0.97, 1.0)	81.0 (70.6, 89.0)	99.8 (99.0, 100.0)	0.99 (0.97, 1.0)
Oxacillin	88.6 (79.5, 94.7)	100.0 (99.3, 100.0)	0.99 (0.98, 1.0)	88.6 (79.5, 94.7)	100.0 (99.3, 100.0)	0.99 (0.98, 1.0)
Rifampicin	80.0 (28.4, 99.5)	99.5 (98.6, 99.9)	0.80 (0.41, 1.0)	60.0 (14.7, 94.7)	99.7 (98.8, 100.0)	0.80 (0.41, 1.0)
Tetracycline	93.4 (87.9, 96.6)	97.1 (95.1, 98.4)	0.95 (0.93, 0.98)	94.1 (88.7, 97.4)	97.3(95.4, 98.6)	0.96 (0.93, 0.98)

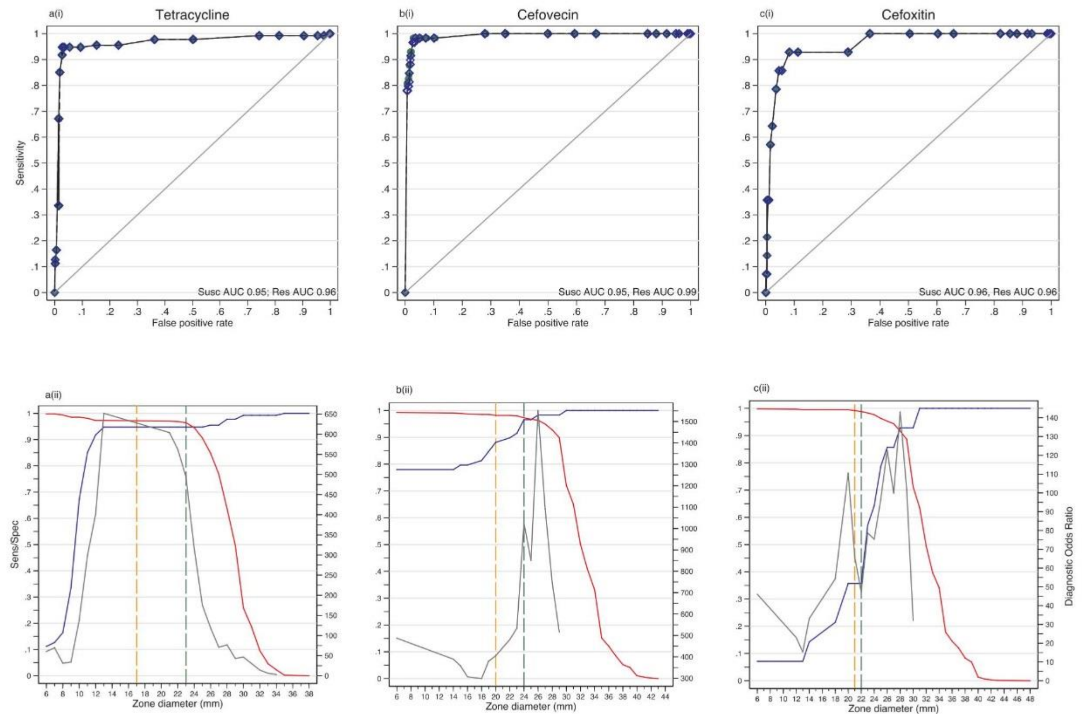

Fig. 1. Receiver-Operating Characteristic (ROC) analysis demonstrating overall performance of disc diffusion relative to broth-microdilution for three selected antimicrobials applied to canine *Staphylococcus pseudintermedius* isolates (n = 614) from clinical cases. ROC plots for tetracycline (a(i)), cefovecin (b (i)), and cefoxitin (c(i)). The green-circle curve is representative of the resistant clinical breakpoint and blue-diamond curve is representative of the susceptible clinical breakpoint for the minimum inhibitory concentration (MIC) as defined by Clinical and Laboratory Standards Institute (CLSI). Diagonal line represents an AUC of 0.5. Two-graph-ROC plots of relative sensitivity (Sens) and relative specificity (Spec) for tetracycline (a(ii)), cefovecin (b(ii)), and cefoxitin (c(ii)). Relative sensitivity (Sens, dash blue line), relative specificity (Spec, solid red line) diagnostic odds ratio (dash-dot grey line), CLIS susceptible breakpoint (green short-dash line) and resistant breakpoint (orange dash line) are plotted on each graph.

38.9%, 64.0%), cefoxitin (35.7%, 95% CI, 12.8%, 64.9%), and cephalothin (43.6%, 95% CI, 27.8%, 60.4%) irrespective of the breakpoint used to dichotomise MIC and zone diameter values.

The accuracy of disc diffusion relative to broth-microdilution, measured as the AUC, was greater than 0.96 for all antimicrobials, except rifampicin (AUC, 0.80) (Table 2). Overall test performance based on ROC analysis (ROC plots and TG-ROC) for three antimicrobials are shown in Fig. 1 (see supplementary materials for ROC plots for other antimicrobials). The ROC plots show minor differences in the accuracy of disc diffusion with the curves for all three antimicrobials approaching the top left-hand corner of the graph. However, the accuracy of disc diffusion varies considerably according to the TG-ROC plots for each antimicrobial evaluated. Disc diffusion is most accurate when the curves for RSe and RSp are close to one at the recommended breakpoints. For tetracycline, both the susceptible and resistant breakpoints correspond to near perfect RSe and RSp estimates, with the DOR indicating disc diffusion has high test discriminatory ability relative to broth-microdilution (Table 3). Drift between RSe and RSp estimates can be seen with cefovecin, with the DOR not at its highest discriminatory ability until sensitivity is over 95%. For cefoxitin, the performance of disc diffusion relative to broth-microdilution is poor when evaluated against both CLSI breakpoints. Here, disc diffusion maximises RSp at the expense of RSe at the recommended cefoxitin breakpoints.

Across all antimicrobials, there was strong diagnostic evidence from positive (resistant) disc diffusion results supporting the presence of resistance as classified by MIC (large LR^+, Table 3). The evidence provided by negative (susceptible) disc diffusion results (small LR^-) was strong using either breakpoint to determine susceptible status. For cefoxitin, disc diffusion was less than accurate at distinguishing susceptible isolates across both breakpoints (LR- = 0.65). The overall discriminatory ability of disc diffusion for all antimicrobials, as assessed by the DOR, was high (DOR > 82) (Table 3).

Observed agreement estimates were > 94.0% for all antimicrobials and breakpoints (Supplementary Tables 1 and 2). Across all antimicrobials and clinical breakpoints, negative percent agreement was > 97% (range 97.1%–99.8%). However, positive percent agreement was much more variable with low values (range, 43.5%–97.1%), particularly for cefoxitin (43.5%, 95% CI 23.2%, 65.5%) and amoxicillin-clavulanic acid (67.3%, 95% CI 57.3%, 76.3%), indicating disagreement between MIC and zone diameter values is associated with the clinical interpretation of resistant (or non-susceptibility) isolates. Antimicrobials with > 1% difference between proportion resistant by broth-microdilution and proportion resistant by disc diffusion recorded statistical significance (mid-*p* McNemar's < 0.05, Supplementary Tables 1 and 2). A higher number of antimicrobials recorded significant mid-*p* McNemar's estimate when the susceptible breakpoint dichotomised MIC and zone diameter values, including amoxicillin-clavulanic acid, cefovecin, cephalothin, and oxacillin. Prevalence-adjusted bias-adjusted Kappa estimates were > 0.9 for all antimicrobials.

The distribution of zone diameters for a selection of four antimicrobials can be appreciated in Fig. 2 (see supplementary materials for histograms of other antimicrobials). Estimates of relative diagnostic accuracy are maximal when there is a clear separation between

Table 3

Estimates of likelihood ratio pairs and diagnostic odds ratios of disc diffusion relative to broth-microdilution for 614 canine *Staphylococcus pseudintermedius* isolates from clinical cases. Clinical and Laboratory Standard Institute (CLSI) susceptible and resistant breakpoints were used to dichotomise minimum inhibitory concentration (MIC) and zone diameter values. LR^+, likelihood ratio of a positive test result; LR^-, likelihood ratio of a negative result, DOR, diagnostic odds ratio. Exact 95% confidence intervals given.

	Susceptible Breakpoint Estimates			Resistant Breakpoint Estimates		
Antimicrobial	LR^+ (95%CI)	LR^- (95%CI)	DOR (95%CI)	LR^+ (95%CI)	LR^- (95%CI)	DOR (95%CI)
Amoxicillin-clavulanic acid	282 (39, 2029)	0.49 (0.38, 0.62)	581 (97, ∞)	282 (9, 2029)	0.49 (0.38, 0.62)	581 (97, ∞)
Cefovecin	77 (32, 186)	0.28 (0.20, 0.40)	274 (103, 726)	59 (20, 118)	0.14 (0.08, 0.27)	407 (153, 108,748)
Cefoxitin	54 (16, 179)	0.65 (0.44, 0.96)	83 (20, 338)	54 (16, 179)	0.65 (0.44, 0.96)	83 (20, 338)
Cephalothin	190 (47, 768)	0.33 (0.22, 0.51)	576 (135, ∞)	251 (34, 1835)	0.57 (0.43, 0.74)	444 (71, ∞)
Chloramphenicol	∞ (117, ∞)	0.15 (0.07, 0.31)	∞ (1671, ∞)	∞ (136, ∞)	0.06 (0.01, 0.21)	∞ (13550, ∞)
Ciprofloxacin	508 (72, 3608)	0.09 (0.04, 0.21)	5580 (768, ∞)	497 (70, 530)	0.12 (0.06, 0.25)	4215 (601, ∞)
Clindamycin	131 (50, 349)	0.01 (0.0, 0.09)	10,692 (1404, ∞)	433 (61, 3080)	0.19 (0.12, 0.30)	2278 (368, ∞)
Oxacillin	∞ (114, ∞)	0.11 (0.06, 0.21)	∞ (1893, ∞)	∞ (114, ∞)	0.11 (0.06, 0.21)	∞ (1893, ∞)
Rifampicin	162 (48, 545)	0.20 (0.03, 1.0)	808 (86, ∞)	183 (39, 868)	0.40 (0.14, 1.0)	455 (57, 3892)
Tetracycline	32 (19, 53)	0.07 (0.04, 0.13)	470 (201, 1102)	35 (20, 59)	0.06 (0.03, 0.12)	569 (233, 1388)

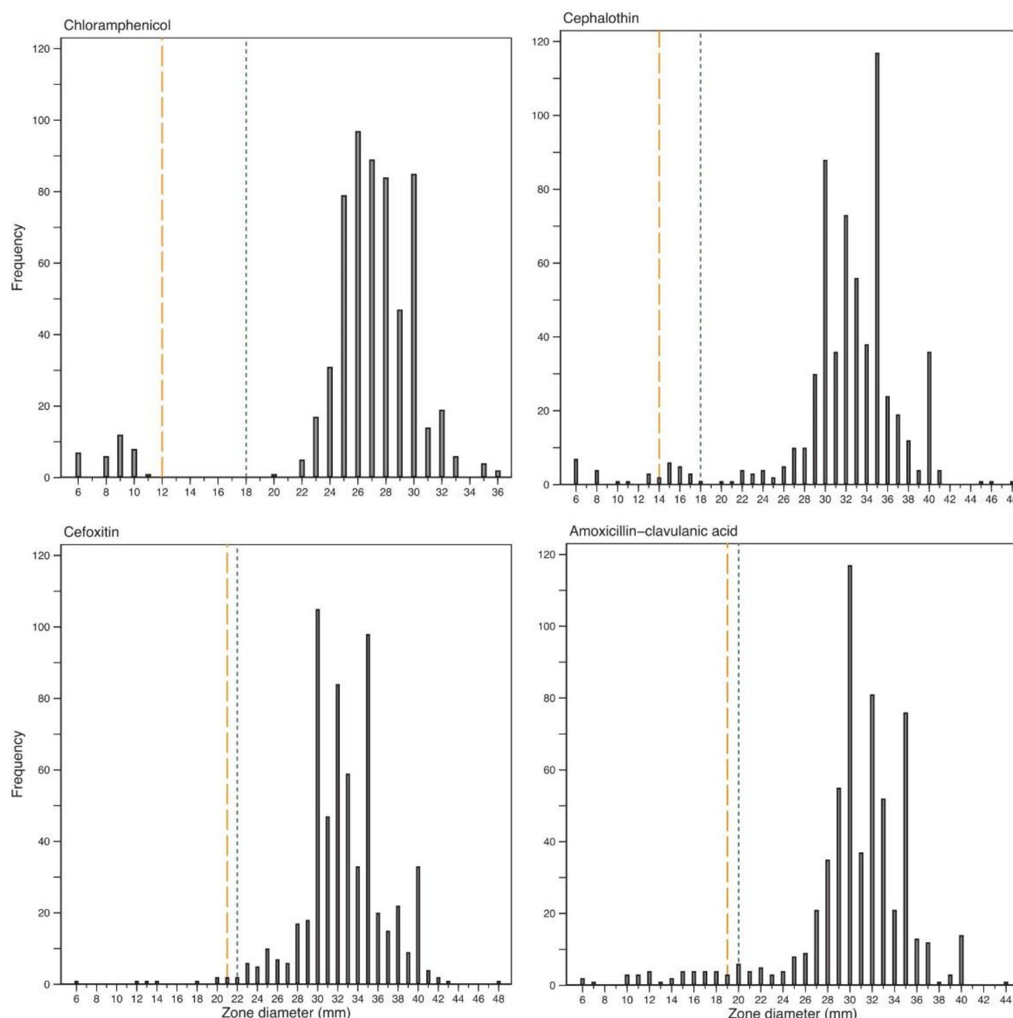

Fig. 2. Distribution of zone diameters for four antimicrobials in canine *Staphylococcus pseudintermedius* isolates (n = 614) from clinical cases. The Clinical and Laboratory Standards Institute (CLSI) susceptible breakpoint (green short-dash) and resistant breakpoint (orange long-dash) is plotted on each distribution.

Table 4
Diagnostic test performance of broth-microdilution and disc diffusion relative to *mecA* real-time PCR for 576 clinical canine *Staphylococcus pseudintermedius* isolates. Clinical and Laboratory Standards Institute (CLSI) susceptible and resistant breakpoints for oxacillin were used to dichotomise minimum inhibitory concentration (MIC) and zone diameter values. RSe, relative diagnostic sensitivity; RSp, relative diagnostic specificity; AUC, area under the curve; LR^+, likelihood ratio of a positive test result; LR^-, likelihood ratio of a negative result, DOR, diagnostic odds ratio. Exact 95% confidence intervals are given.

Test Performance estimates	Broth-microdilution (95% CI)	Disc Diffusion (95% CI)
RSe (%)	96.1 (88.9, 99.2)	86.8 (77.1, 93.5)
RSp (%)	99.6 (98.6, 100.0)	99.8 (98.9, 100.0)
AUC	0.99 (0.97, 1.00)	0.99 (0.96, 1.00)
LR+	240 (60.2, 958)	434 (61.2, 3082)
LR-	0.04 (0.01, 0.12)	0.13 (0.07, 0.24)
DOR	6059 (1059, 33,932)	3293 (511, ∞)
Oxacillin resistance (%)	13.0 (10.4, 16.1)	11.6 (9.1, 14.5)
Observed Agreement (%)	99.1 (98.0, 99.7)	98.1 (96.6, 99.0)
McNemars mid-*p* value	1.0	0.01

populations as demonstrated on the zone diameter histogram for chloramphenicol. However, performance estimates were imperfect when populations overlap and, or breakpoints were close together (*e.g.*, cefoxitin, cephalothin, amoxicillin-clavulanic acid, Fig. 2). Moreover, estimates of relative accuracy may be imprecise when the range of zone diameters is narrow (*i.e.*, mostly all susceptible), as can be seen for cefoxitin and rifampicin (supplementary materials).

4.2. Broth-microdilution and disc diffusion performance relative to mecA real-time PCR

For the evaluation of broth-microdilution and disc diffusion relative to *mecA* real-time PCR, 576 isolates were evaluated. In total, 13.2% (n = 76) of isolates were *mecA*-positive by real-time PCR, while 13.0% (n = 75) were oxacillin resistant by broth-microdilution and 11.6% (n = 67) by disc diffusion (Table 4). The relative diagnostic test accuracy of broth-microdilution and disc diffusion was high (AUC > 0.99). DOR estimates reflect the strong RSe and RSp estimates for both assays, however, disc diffusion's RSe was significantly lower than broth-microdilution (McNemars mid-*p* value < 0.01). There was a significant difference between the proportion of isolates identified as oxacillin-resistant by disc diffusion and by *mecA* real-time PCR (McNemars mid-*p* value < 0.01, Table 4). The overlap between oxacillin-susceptible isolates by disc diffusion and *mecA* positive status can be seen in Fig. 3. An overall comparison of the ROC analysis demonstrated negligible difference in the accuracy of broth-microdilution and disc diffusion. However, the robustness of the assays varies according to their corresponding TG-ROC plots (Fig. 3). For both assays, small movements in clinical breakpoints (and zone measurements for decreased susceptible isolates) will result in a noticeable change in RSe and RSp, especially so for disc diffusion.

4.3. mecA sequence analysis

Nine isolates identified as phenotypically susceptible to oxacillin by disc diffusion (9/9) or broth microdilution (3/9) and *mecA* positive on real-time PCR underwent whole-genome sequencing. Details of phenotypic and genotypic characteristics of the isolates are detailed in Supplementary Table 3. Six strain types were identified including two from the same sequence type, ST498. Two strains were from new sequence types. The nine isolates were confirmed *mecA* positive with 99.5%–100% identity and 100% full length coverage and all contained *blaZ* or *blaZ*-like elements.

5. Discussion

The main finding from this study is the high level of accuracy of disc diffusion relative to broth-microdilution for most antimicrobials evaluated in clinical *S. pseudintermedius* when performed according to CLSI guidelines. This finding holds regardless of the CLSI interpretative criteria used to evaluate the performance of disc diffusion. For clindamycin, an antibiotic commonly recommended as a first-line treatment for *S. pseudintermedius* infections in dogs (Hillier et al., 2014), the accuracy of disc diffusion was comparable to broth-microdilution. Similarly, disc diffusion was accurate for cefovecin; an antibiotic often recommended as a final treatment option. However, the accuracy of disc diffusion was unsatisfactory for amoxicillin-clavulanic acid and cephalothin, antimicrobials that are also frequently used to *S. pseudintermedius* infections. For these antimicrobials, disc diffusion has limitations when determining the phenotypic susceptibility of *S. pseudintermedius*.

These findings are of concern for amoxicillin-clavulanic acid which is widely used to treat skin infections in dogs as there are no published veterinary-specific zone diameter interpretative criteria for *S. pseudintermedius*, and CLSI removed zone diameter clinical breakpoints for all antistaphylococcal beta-lactams in 2012 (Dien Bard et al., 2014). Thus, the amoxicillin-clavulanic acid zone diameter breakpoints listed in older versions of CLSI document VET01 are likely to be unreliable. Considering this, and the findings from this study, veterinary laboratories that evaluate the susceptibility of clinical *S. pseudintermedius* isolates to amoxicillin-clavulanic acid using the disc diffusion assay are advised to discontinue the practice. Furthermore, veterinary laboratories are advised to limit disc diffusion testing of clinical *S. pseudintermedius* isolates to oxacillin and penicillin to infer the susceptibility for other beta-lactam antimicrobial agents, except for newer cephalosporins with anti-MRSA activity. These recommendations are consistent with CLSI (2018), and findings reported by Dien Bard et al. (2014) and Siak et al. (2014). This study also provides quantitative and graphical evidence to confirm the inadequacy of disc diffusion testing for determining the susceptibility of *S. pseudintermedius* to cefoxitin. Given the potential for a high number of misclassification errors, cefoxitin should not be included in the panel of antibiotics used to evaluate antimicrobial susceptibility of animal-derived *S. pseudintermedius*.

Broth-microdilution and disc diffusion were shown to be relatively comparable at predicting the presence of the *mecA* gene in clinical *S. pseudintermedius* isolates. For both assays, predicting of the absence of the *mecA* gene was high, while broth-microdilution out-performed disc diffusion when predicting the presence of the *mecA* gene. Other studies have also found phenotypic oxacillin resistance is a reliable predictor of the presence of the *mecA* gene in *S. pseudintermedius* (Bemis et al., 2009; Schissler et al., 2009; Worthing et al., 2018a; Wu et al., 2016). In this study, nine isolates were identified as oxacillin-susceptible by disc diffusion or broth-microdilution yet harboured the *mecA* gene. A similar observation has been reported in other studies (Eckholm et al., 2013; Feng et al., 2012; Griffeth et al., 2008; Kania et al., 2004; Kuwahara-Arai et al., 1996). It has been suggested that failure to express the PBP2a protein may be due to mutation, down-regulation or suppression of *mecA* gene expression (Kania et al., 2004; Kuwahara-Arai et al., 1996). The type of staphylococcal cassette chromosome that harbours the *mecA* gene (SCC*mec*) has also been shown to affect oxacillin MIC in MRSP isolates (Kasai et al., 2016; Worthing et al., 2018b). The collection of *mecA*-positive, oxacillin-susceptible isolates included isolates from ST498, ST539 and ST547. These sequence types were previously shown to harbour SCC*mec* types IVg and NA45, both of which have significantly lower oxacillin MIC values than other SCC*mec* types (Worthing et al., 2018b). Heterogeneous resistance, where there is existence of susceptible and resistant organisms within a single strain has also been proposed (Kania et al., 2004; Savini et al., 2013). Variations in salinity, temperature, pH, or the presence of beta-lactam during laboratory culture is also reported to have an effect on phenotype

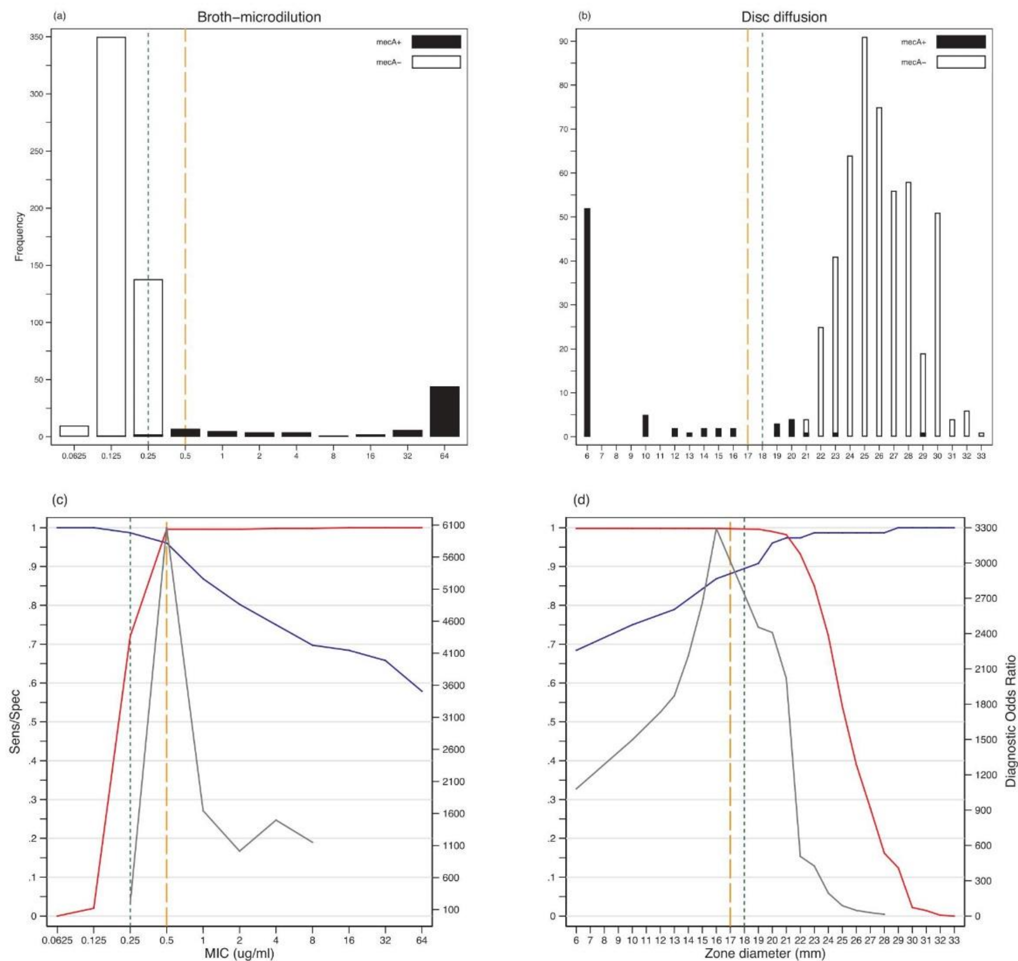

Fig. 3. Diagnostic test performance attributes for broth-microdilution and disc diffusion for oxacillin in canine *Staphylococcus pseudintermedius* isolates (n = 576) from clinical cases. (a) Minimum inhibitory concentration (MIC) values by broth-microdilution, and (b) zone diameter values by disc diffusion are compared to *mecA* real-time PCR status. (c) Two-graph Receiver-Operating Characteristic (TG-ROC) plots for broth-microdilution, and (d) disc diffusion, plot the relative sensitivity (Sens), relative specificity (Spec), and diagnostic odds ratio (DOR). Real-time *mecA* PCR is the reference test. Relative sensitivity (Sens, dash blue solid line), relative specificity (Spec, solid red line), diagnostic odds ratio (dash-dot grey line). Clinical and Laboratory Standards Institute (CLSI) susceptible (green short dash line) and resistant (orange long-dash line) breakpoints are plotted on all graphs.

expression (Griffeth et al., 2008). Additionally, the precision of measurements derived from the phenotypic assays for decreased susceptible isolates must also be considered. For example, six of the nine isolates identified as oxacillin-susceptible by disc diffusion were within 3 mm of the resistant clinical breakpoint, while five of the nine MICs were equal to the resistant clinical breakpoint. The detection of isolates with decreased susceptibility depends very much on the validity of the assay, the proficiency of technicians, and the breakpoint used to classify the isolates.

In parallel with an earlier study involving *Escherichia coli* (Badger et al., 2018), we report a broad range of measurements for disc diffusion applied to *S. pseudintermedius*. Quantitative estimates from large studies of these kind are generally lacking in veterinary literature for a broad range of diagnostic tests. Evidenced-based clinical decisions are better supported with robust estimates of test accuracy. Also, in surveillance settings, diagnostic sensitivity and specificity can be used in standard equations to adjust the apparent prevalence of disc diffusion, thereby allowing direct comparison with ('true') prevalence measured by broth-microdilution. ROC analysis is useful to determine test accuracy and assist in defining breakpoint values since it is independent of prevalence in a population. Depending on the purpose of the test and the clinical or epidemiological setting, either diagnostic sensitivity or specificity can be improved by altering the breakpoint used to define resistance.

While this study was comprised of a comprehensive collection of clinical *S. pseudintermedius* isolates submitted from all veterinary laboratories in Australia over one year, it cannot be considered a broad representation of all clinical cases presented to primary care veterinarians. Findings from this study may be biased towards the inclusion of resistant isolates since veterinarians are more likely to submit samples from cases that may have already failed initial treatment. Biases arising from selective inclusion of animals or isolates in studies are well recognised (Lash et al., 2014; Laupland et al., 2007). Ciprofloxacin was used as a representative of the fluoroquinolone class as it is commonly

used in national surveillance owning to its relevance to public health. However, there is a need to evaluate the performance of other fluoroquinolone class members specific to animal health, such as enrofloxacin and marbofloxacin, for clinical decision-making. Data for this study were generated in two reference laboratories (University of Adelaide, Murdoch University) and may not reflect the variation in results which may occur when multiple primary laboratories perform phenotypic assays. Moreover, since broth-microdilution is an imperfect reference test the performance estimates for disc diffusion reported here can never exceed those of broth-microdilution.

6. Conclusion

Overall, this study demonstrates that for most antimicrobials evaluated, disc diffusion can be used to differentiate veterinary *S. pseudintermedius* isolates that might otherwise be assessed by broth-microdilution when performed according to CLSI guidelines. However, disc diffusion performance was less favourable for amoxicillin-clavulanic acid, cephalothin, and cefoxitin. Therefore, veterinary laboratories are advised to use oxacillin or penicillin to infer phenotypic susceptibility of *S. pseudintermedius* to other beta-lactam antibiotics, except for newer anti-MRSA cephalosporins. We found disc diffusion and broth-microdilution approximated genotypic results from *mecA* real-time PCR when using oxacillin to predict methicillin resistance, and there was minimal difference in the performance estimates between both phenotypic assays relative to PCR. These findings demonstrate that disc diffusion susceptibility data from clinical *S. pseudintermedius* could be acquired for national surveillance provided consideration is given to the diagnostic test performance estimates reported here.

Declaration of interest statement

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Ethical approval

None required.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.05.024.

References

Abraham, S., Chin, J., Brouwers, H.J., Zhang, R., Chapman, T.A., 2012. Molecular serogrouping of porcine enterotoxigenic Escherichia coli from Australia. J. Microbiol. Methods 88, 73–76.

Abraham, S., Kirkwood, R.N., Laird, T., Saputra, S., Mitchell, T., Singh, M., Linn, B., Abraham, R.J., Pang, S., Gordon, D.M., Trott, D.J., O'Dea, M., 2018. Dissemination and persistence of extended-spectrum cephalosporin-resistance encoding IncI1-blaCTXM-1 plasmid among Escherichia coli in pigs. ISME J. 12, 2352–2362.

Badger, S., Abraham, S., Saputra, S., Trott, D.J., Turnidge, J., Mitchell, T., Caraguel, C.G.B., Jordan, D., 2018. Relative performance of antimicrobial susceptibility assays on clinical Escherichia coli isolates from animals. Vet. Microbiol. 214, 56–64.

Bemis, D.A., Jones, R.D., Frank, L.A., Kania, S.A., 2009. Evaluation of susceptibility test breakpoints used to predict mecA-mediated resistance in Staphylococcus pseudintermedius isolated from dogs. J. Vet. Diagn. Investig. 21, 53–58.

Bemis, D.A., Jones, R.D., Videla, R., Kania, S.A., 2012. Evaluation of cefoxitin disk diffusion breakpoint for detection of methicillin resistance in Staphylococcus pseudintermedius isolates from dogs. J. Vet. Diagn. Investig. 24, 964–967.

CLSI, 2013a. Performance Standards for Antimicorbial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals; (VET01-S2), 2nd ed. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI, 2013b. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals (VET01-A4), 4th ed. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI, 2015. Performance standards for antimicrobial susceptibility testing; (M100-S25). 25th Informational Supplement. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI, 2018. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals (VET08), 4th ed. Clinical and Laboratory Standards Institute, Wayne, PA.

Costa, A.M., Kay, I., Palladino, S., 2005. Rapid detection of mecA and nuc genes in staphylococci by real-time multiplex polymerase chain reaction. Diagn. Microbiol. Infect. Dis. 51, 13–17.

Dien Bard, J., Hindler, J.A., Gold, H.S., Limbago, B., 2014. Rationale for eliminating Staphylococcus breakpoints for beta-lactam agents other than penicillin, oxacillin or cefoxitin, and ceftaroline. Clin. Infect. Dis. 58, 1287–1296.

Eckholm, N.G., Outerbridge, C.A., White, S.D., Sykes, J.E., 2013. Prevalence of and risk factors for isolation of meticillin-resistant Staphylococcus spp. From dogs with pyoderma in northern California. USA. Vet Dermatol 24 (154-161), e134.

Fagerland, M.W., Lydersen, S., Laake, P., 2013. The McNemar test for binary matched-pairs data: mid-p and asymptotic are better than exact conditional. BMC Med. Res. Methodol. 13, 91.

Feng, Y., Tian, W., Lin, D., Luo, Q., Zhou, Y., Yang, T., Deng, Y., Liu, Y.-H., Liu, J.-H., 2012. Prevalence and characterization of methicillin-resistant Staphylococcus pseudintermedius in pets from South China. Vet. Microbiol. 160, 517–524.

Glas, A.S., Lijmer, J.G., Prins, M.H., Bonsel, G.J., Bossuyt, P.M., 2003. The diagnostic odds ratio: a single indicator of test performance. J. Clin. Epidemiol. 56, 1129–1135.

Greiner, M., Gardner, I.A., 2000. Epidemiologic issues in the validation of veterinary diagnostic tests. Prev. Vet. Med. 45, 3–22.

Griffeth, G.C., Morris, D.O., Abraham, J.L., Shofer, F.S., Rankin, S.C., 2008. Screening for skin carriage of methicillin-resistant coagulase-positive staphylococci and Staphylococcus schleiferi in dogs with healthy and inflamed skin. Vet. Dermatol. 19, 142–149.

Hardefeldt, L.Y., Marenda, M., Crabb, H., Stevenson, M.A., Gilkerson, J.R., Billman-Jacobe, H., Browning, G.F., 2018. Antimicrobial susceptibility testing by Australian veterinary diagnostic laboratories. Aust. Vet. J. 96, 142–146.

Hillier, A., Lloyd, D.H., Weese, J.S., Blondeau, J.M., Boothe, D., Breitschwerdt, E., Guardabassi, L., Papich, M.G., Rankin, S., Turnidge, J.D., Sykes, J.E., 2014. Guidelines for the diagnosis and antimicrobial therapy of canine superficial bacterial folliculitis (Antimicrobial guidelines working group of the international society for companion animal infectious diseases). Vet. Dermatol. 25 (163-175), e142–163.

Kania, S.A., Williamson, N.L., Frank, L.A., Wilkes, R.P., Jones, R.D., Bemis, D.A., 2004. Methicillin resistance of staphylococci isolated from the skin of dogs with pyoderma. Am. J. Vet. Res. 65, 1265–1268.

Kasai, T., Saegusa, S., Shirai, M., Murakami, M., Kato, Y., 2016. New categories designated as healthcare-associated and community-associated methicillin-resistant Staphylococcus pseudintermedius in dogs. Microbiol. Immunol. 60, 540–551.

Kuwahara-Arai, K., Kondo, N., Hori, S., Tateda-Suzuki, E., Hiramatsu, K., 1996. Suppression of methicillin resistance in a mecA-containing pre-methicillin-resistant Staphylococcus aureus strain is caused by the mecI-mediated repression of PBP 2′ production. Antimicrob. Agents Chemother. 40, 2680–2685.

Lash, T.L., Fox, M.P., MacLehose, R.F., Maldonado, G., McCandless, L.C., Greenland, S., 2014. Good practices for quantitative bias analysis. Int. J. Epidemiol. 43, 1969–1985.

Laupland, K.B., Ross, T., Pitout, J.D., Church, D.L., Gregson, D.B., 2007. Investigation of sources of potential bias in laboratory surveillance for anti-microbial resistance. Clin. Invest. Med. 30, E159–166.

Nakagawa, S., Taneike, I., Mimura, D., Iwakura, N., Nakayama, T., Emura, T., Kitatsuji, M., Fujimoto, A., Yamamoto, T., 2005. Gene sequences and specific detection for Panton-Valentine leukocidin. Biochem. Biophys. Res. Commun. 328, 995–1002.

O'Dea, M.A., Laird, T., Abraham, R., Jordan, D., Lugsomya, K., Fitt, L., Gottschalk, M., Truswell, A., Abraham, S., 2018. Examination of Australian Streptococcus suis isolates from clinically affected pigs in a global context and the genomic characterisation of ST1 as a predictor of virulence. Vet. Microbiol. 226, 31–40.

Saputra, S., Jordan, D., Worthing, K.A., Norris, J.M., Wong, H.S., Abraham, R., Trott, D.J., Abraham, S., 2017. Antimicrobial resistance in coagulase-positive staphylococci isolated from companion animals in Australia: a one year study. PLoS One 12, e0176379.

Savini, V., Di Giuseppe, N., Fazii, P., D'Amario, C., D'Antonio, D., Carretto, E., 2013. Staphylococcus pseudintermedius heterogeneously expresses the mecA gene. Vet. Microbiol. 165, 489–490.

Schissler, J.R., Hillier, A., Daniels, J.B., Cole, L.K., Gebreyes, W.A., 2009. Evaluation of Clinical Laboratory Standards Institute interpretive criteria for methicillin-resistant Staphylococcus pseudintermedius isolated from dogs. J. Vet. Diagn. Invest.: Off. Publ. Am. Assoc. Vet. Lab. Diagn., Inc 21, 684–688.

Schmidt, V.M., Williams, N.J., Pinchbeck, G., Corless, C.E., Shaw, S., McEwan, N., Dawson, S., Nuttall, T., 2014. Antimicrobial resistance and characterisation of staphylococci isolated from healthy Labrador retrievers in the United Kingdom. BMC Vet. Res. 10, 17.

Siak, M., Burrows, A.K., Coombs, G.W., Khazandi, M., Abraham, S., Norris, J.M., Weese, J.S., Trott, D.J., 2014. Characterization of meticillin-resistant and meticillin-

susceptible isolates of Staphylococcus pseudintermedius from cases of canine pyoderma in Australia. J. Med. Microbiol. 63, 1228–1233.
van Duijkeren, E., Catry, B., Greko, C., Moreno, M.A., Pomba, M.C., Pyorala, S., Ruzauskas, M., Sanders, P., Threlfall, E.J., Torren-Edo, J., Torneke, K., 2011. Review on methicillin-resistant Staphylococcus pseudintermedius. J. Antimicrob. Chemother. 66, 2705–2714.
Worthing, K.A., Abraham, S., Coombs, G.W., Pang, S., Saputra, S., Jordan, D., Trott, D.J., Norris, J.M., 2018a. Clonal diversity and geographic distribution of methicillin-resistant Staphylococcus pseudintermedius from Australian animals: discovery of novel sequence types. Vet. Microbiol. 213, 58–65.
Worthing, K.A., Schwendener, S., Perreten, V., Saputra, S., Coombs, G.W., Pang, S., Davies, M.R., Abraham, S., Trott, D.J., Norris, J.M., 2018b. Characterization of staphylococcal cassette chromosome mec elements from methicillin-resistant Staphylococcus pseudintermedius infections in australian animals. mSphere 3.
Wu, M.T., Burnham, C.A., Westblade, L.F., Dien Bard, J., Lawhon, S.D., Wallace, M.A., Stanley, T., Burd, E., Hindler, J., Humphries, R.M., 2016. Evaluation of Oxacillin and Cefoxitin Disk and MIC Breakpoints for Prediction of Methicillin Resistance in Human and Veterinary Isolates of Staphylococcus intermedius Group. J. Clin. Microbiol. 54, 535–542.

Supplementary materials for Chapter 3 can be found in Appendix 2

Chapter 4:

Intra- and inter-laboratory agreement of the disc diffusion assay for assessing antimicrobial susceptibility of porcine *Escherichia coli*

Contextual Statement

In Chapters 2 and 3, the accuracy of disc diffusion relative to broth microdilution was found to be satisfactory for determining susceptibility in clinical *Escherichia coli* and *S. pseudintermedius* for most antimicrobial agents evaluated. The other component to evaluating diagnostic performance is to determine an assay's precision. Understanding measurement imprecision (i.e., variability) in a diagnostic test is critical not only for clinical interpretation but also for determining whether a diagnostic test is suitable for use in surveillance activities. Hence, in Chapter 4, the precision of disc diffusion was investigated to determine the extent of variation in measurements that can be expected when the test is performed in veterinary diagnostic laboratories. A test-retest study design was used to determine intra-laboratory agreement (repeatability) and inter-laboratory agreement (reproducibility). Repeatability and reproducibility estimates provide a practical interpretation of the extent of variation in zone diameter measurements expected in veterinary laboratories when testing the same isolate. Seven veterinary diagnostic laboratories participated in the study and tested replicates from the same twenty clinical *E. coli* isolates from pigs five times over time. The findings from this study, coupled with those from Chapters 2 and 3, will help determine whether antimicrobial susceptibility data from disc diffusion can be acquired from veterinary laboratories for use in national surveillance programs.

Statement of Authorship

Title of Paper	Intra- and inter-laboratory agreement of the disc diffusion assay for assessing antimicrobial susceptibility of porcine *Escherichia coli*
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Principal Author

Name of Principal Author (Candidate)	Skye Badger		
Contribution to the Paper	Study design, performed analysis, interpreted data, wrote manuscript, corresponding author.		
Overall percentage (%)	85		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	3/5/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Supervised work, assisted with study design, data interpretation, manuscript evaluation		
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Contribution to the Paper	Supervised work, assisted with study design, statistical modelling and data interpretation, stata coding, manuscript evaluation		
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Contribution to the Paper	Supervised work, assisted with study design, statistical modelling and data interpretation, stata coding, manuscript evaluation		
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Intra- and inter-laboratory agreement of the disc diffusion assay for assessing antimicrobial susceptibility of porcine *Escherichia coli*

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ABSTRACT

Reliable assessment of the susceptibility of animal bacterial pathogens to antimicrobials is of paramount importance in the fight against antimicrobial resistance. This work aims to estimate the repeatability (intra-laboratory agreement) and reproducibility (inter-laboratory agreement) of the disc diffusion assay in veterinary laboratories to understand further if the assay has a role in the surveillance of antimicrobial resistance in animals. Seven major veterinary laboratories from all States in Australia participated, and each tested the same panel of isolates five times at three to four-week intervals, against six antimicrobial agents using Clinical and Laboratory Standards Institute protocols. The panel consisted of twenty different isolates from porcine *Escherichia coli* from clinical cases and a single reference strain (ATCC 25922). Laboratories were blinded to the identity of the isolates, replicates, and to each other. In total, 4200 inhibition zone diameters (mm) were collected, and analysed descriptively, graphically, and with linear mixed models. Regardless of the laboratories and isolate/antimicrobial combinations, the overall very major error rate (proportion of isolates classified as susceptible when actual status is resistant) was 1.6%; the major error rate (proportion of isolates classified as resistant when actual status is susceptible) was 1.6%; and the 'minor error' rate (proportion of isolates with intermediate susceptibility that measure fully susceptible or resistant or vice versa) was 2.4%. The variation between repeated measurements ranged between 4.4–7.2 mm depending on the antimicrobial agent assessed. The reproducibility was always more variable than the repeatability, which suggested some laboratory effects. The repeatability coefficient of disc diffusion was lowest for tetracycline (4.4 mm, 95% CI: 3.8–5.0 mm) and ampicillin (4.6 mm, 95% CI: 4.2–5.2 mm) and highest for trimethoprim-sulfamethoxazole (6.6 mm, 95% CI: 5.9–7.4 mm). The reproducibility coefficient of disc diffusion was lowest for gentamicin (5.4, 95% CI: 4.0–7.2) and highest for trimethoprim-sulfamethoxazole (7.2 mm, 95%CI: 4.5–11.7 mm). The precision of the disc diffusion assay was deemed satisfactory for use in a national surveillance program for clinical porcine *E. coli* isolates. However, measurement variation of the disc diffusion assay is of concern for isolates with marginal susceptibility or resistance due to increased risk of misclassification.

1. Introduction

Integral to the success of any surveillance program is the performance of the diagnostic assays used to detect the disease of interest (OIE, 2018a). Factors which influence assay performance need to be understood for the practical interpretation of assay results, and to

Abbreviations: ATCC, American Type Culture Collection; CLSI, Clinical Laboratory Standards Institute; ECOFF, epidemiological cut-off value; EUCAST, European Centre for Antimicrobial Susceptibility Testing; ISO, International Standards Organisation; OIE, World Organisation for Animal Health

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guide the response to a disease threat. The International Standards Organisation (ISO) outlines the general principles required for the validation of diagnostic tests (ISO, 1994) including methods for determining a test's accuracy and precision. While accuracy refers to the deviation of a measurement from the 'true' value of the subject, precision reflects the closeness of measurements to each other (Gerke et al., 2016). Estimates of precision are used to determine whether single measurements reported by multiple observers (such as laboratories) can be used interchangeably (Barnhart et al., 2007), an important consideration when data may be acquired from multiple laboratories for use in national surveillance (OIE, 2018b).

In literal terms, repeated measurements agree if they are identical. However, repeated measurements made on a continuous scale are less likely to agree because they are more susceptible to inherent random errors (Vaz et al., 2013). Some error in measurement values can be acceptable, depending on the context and the impact of the error (Barnhart et al., 2007). For instance, a measurement error may be acceptable if it lies within a specified range of values, for example, when validating an assay using standardised quality control strains. On the other hand, measurement error may be less acceptable if the measurement value is close to a clinical breakpoint used in therapeutic decision-making. Agreement of an assay can be estimated at two different levels – repeatability and reproducibility. Repeatability is assessed under similar, if not identical, analytical conditions (e.g., same laboratory), whereas reproducibility is assessed under varying analytical conditions (e.g., different laboratories) (ISO, 1994). The interest in reproducibility studies lies in the comparison of repeated measurements by different laboratories (Bartlett and Frost, 2008), making these estimates particularly useful when inferences are to be made on the broader population, such as veterinary laboratories operating in a national network. Notably, measures of agreement should not be considered in isolation to measures of diagnostic accuracy since it is possible to have a test with excellent precision yet be systematically biased (i.e., measurements deviate from the true value) and vice versa (Jordan et al., 2012; Vaz et al., 2013).

Disc diffusion is the most widely used phenotypic antimicrobial susceptibility assay performed in veterinary microbiology laboratories (Dargatz et al., 2017; Hombach et al., 2017). The assay is generally considered reliable provided it is performed according to international standards such as those published by the Clinical and Laboratory Standards Institute (CLSI) and the European Centre for Antimicrobial Susceptibility Testing (EUCAST) (Lestari et al., 2008; Matuschek et al., 2014). However, even when international standards are adhered to, problems intrinsic to the disc diffusion assay, such as variation in the agar, inoculum, and manual measurement of zone-diameters, have been reported (Murray et al., 1982; Nijs et al., 2003; Hombach et al., 2017). The usefulness of the disc diffusion assay for clinical decision-making and use in national antimicrobial resistance surveillance programs is dependent on it having robust repeatability and reproducibility characteristics (Murray et al., 1982). Studies have assessed the precision of the disc diffusion assay based on the evaluation of quality control strains recommended by CLSI or EUCAST (Murray et al., 1982; Hombach et al., 2013; Matuschek et al., 2014), or in the course of comparing methods for measuring zone-diameters (Medeiros and Crellin, 2000; Lehtopolku et al., 2012; Idelevich et al., 2016; Hombach et al., 2017). However, no studies have reported repeatability and reproducibility estimates for the assay when performed on pathogenic strains from clinical submissions.

By evaluating the precision of the disc diffusion assay using pathogenic strains, this study seeks to replicate the routine testing that occurs in veterinary diagnostic laboratories and the biological variation that can occur in strains which are not as predictable as quality control strains. Therefore, the purpose of this study was to determine the repeatability and reproducibility of the disc diffusion assay in veterinary diagnostic laboratories for pathogenic *Escherichia coli* isolates derived from diseased pigs. *Escherichia coli* isolates derived from pigs were chosen in this study as they are a key bacterial isolate monitored in all national antimicrobial resistance surveillance programs. The study is reported under the Guidelines for Reporting Reliability and Agreement Studies (GRRAS) (Kottner et al., 2011).

2. Methods and materials

2.1. Study design

The study was designed to repeatedly measure the zone diameter of the same *E. coli* isolates across seven participating Australian veterinary laboratories over time. Participation in the study was conditional on the laboratory agreeing to perform the disc diffusion assay according to CLSI protocols. A nominal sum (AUD 600) was offered to participating laboratories to offset direct costs arising from the consumption of laboratory reagents. Participants were not advised to use preferred laboratory reagents during the study in order to replicate routine testing procedures in each laboratory.

Participating laboratories received five replicated batches of 20 *E. coli* isolates three to four weeks apart (a total of 100 *E. coli* replicates per laboratory). Laboratories were requested to process each batch at reception and report results via an online reporting form within ten working days. Participating laboratories were blinded to the identity of the isolates between batches, replicates within batches, and to each other. Other than the first author, all other authors were blinded to the identity of participating laboratories. In this study, participating laboratories are referred to by their randomly assigned letter (A to G) to protect their identity.

2.2. Selection of isolates

Pathogenic *E. coli* isolates used in this study comprised nineteen isolates from laboratory submissions of diseased pigs' specimens plus one CLSI quality control strain for *E. coli*, ATCC 25922. Fifteen of nineteen isolates were randomly selected (using a computer-generated algorithm) from a previously described national collection of pathogenic *E. coli* isolates from pigs (n = 324) (Abraham et al., 2015). While four isolates were purposely selected for their resistance to ceftiofur, a rare occurrence in *E. coli* isolates from pigs in Australia (Abraham et al., 2015). (See Supplementary Table 1 for original reference laboratory identification of isolates included in this study). The *E. coli* isolates were kept at- 80 °C storage at the Antimicrobial Resistance and Infectious Diseases Laboratory at Murdoch University, Perth until processing. Isolates were assigned a random identification number from one to 20, with the first 15 numbers reserved for the randomly selected *E. coli* isolates. Each replicate for each isolate was assigned a unique six-digit code generated at random by a computer algorithm. Participating laboratories were informed that all isolates were confirmed veterinary *E. coli* strains, however, animal-species and sampling site were not disclosed.

2.3. Preparation of replicates

Each isolate was sub-cultured twice on 5% sheep-blood agar (Edwards, Australia) and frozen at - 80 °C in individual aliquots containing brain-heart infusion broth with 20% glycerol. All isolates underwent confirmatory identification using Matrix-Assisted Laser Desorption Ionisation Time of Flight mass spectrometry (MALDI-TOF MS, Bruker Daltonik, Germany). In preparation for each batch (submission), partially thawed aliquots of the isolates were sub-cultured twice on 5% sheep-blood agar as per CLSI procedures. Individual swabs were taken from the agar plate of each isolate using Aimes charcoal agar gel transport media (Copan Diagnostics, CA) and dispatched by overnight courier to the seven laboratories. Each swab was labelled with a unique six-digit identification code.

2.4. Disc diffusion method

Participating laboratories were instructed to conduct disc diffusion testing according to either CLSI performance standards M02-A12 (CLSI, 2015a) or VET01-A4 (CLSI, 2013). Disc diffusion testing was to be performed under routine laboratory conditions, and laboratories were instructed not to conduct additional confirmatory testing of the isolates. A panel of six antimicrobial discs was specified: ampicillin (10 µg), ceftiofur (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), tetracycline (30 µg), and trimethoprim-sulfamethoxazole (1.25/23.7 µg). Participating laboratories recorded zone-diameter measurements (mm) and clinical interpretation for each isolate/antimicrobial combination in a standardised online reporting form designed in Qualtrics survey software (www.qualtrics.com.au). Date of assessment and the method used to measure the zone-diameters was recorded.

2.5. Descriptive analysis

At the completion of the study, results were downloaded from the Qualtrics survey software platform as a comma-separated value file format and imported into Stata version 15.1 (Stata Corporation, College Station, TX) for analysis. A Stata program was written to de-code the six-digit identity assigned to each replicate so repeated zone-diameter measurements could be matched to isolate and laboratory for analysis.

Standard measures of central tendency and variability for each isolate/ antimicrobial combination were summarised, including the mean, median, standard deviation, and minimum and maximum values. Graphical analyses were performed by grouping zone-diameter measurements by isolate, antimicrobial, and laboratory to illustrate variation among these factors. Zone-diameter measurements were categorised using CLSI susceptible and resistant clinical breakpoints (CLSI, 2015b, 2018) and epidemiological cut-off (ECOFF) values (Table 1). Deviation was recorded as (i) a 'very major error' when an individual zone-diameter measurement indicated susceptibility and the median value of all zone-diameter measurements for the isolate/ antimicrobial combination indicated resistance (false-susceptibility); (ii) 'major error' when an individual zone-diameter indicated resistance and the median value of all zone-diameter measurements for the isolate/ antimicrobial combination indicated susceptibility (false-resistance); or (iii) a 'minor error' when an individual zone-diameter was intermediate, and the median value of all zone-diameter measurements for the isolate/ antimicrobial combination was either susceptible or resistant and vice versa.

The within-isolate coefficient of variation (CV) estimates was calculated as the crude standard deviation of repeated zone-diameter measurements divided by the mean of zone-diameter measurements for each isolate/antimicrobial combination. The CV is a measure of relative variability, which reflects the relative amplitude of the variability between measurements on the same isolate/ antimicrobial combination (OIE, 2018c). The 95% confidence intervals for the CV estimates were estimated using the bias-corrected accelerated level bootstrapping estimation (Efron, 1987).

2.6. Estimation of repeatability and reproducibility of disc diffusion

A linear mixed model was fitted for each antimicrobial to estimate the relative contributions of different factors (laboratory, isolate, batch, and residuals) to the total variability in zone-diameter measurements. Estimates of variance were obtained from the *mixed* command in Stata and used to calculate the repeatability and reproducibility for each antimicrobial. In this study, repeatability coefficient (r) refers to the 95% expected variation between two zone-diameter measurements within a laboratory while reproducibility coefficient (R) refers to the 95% expected variation of two zone-diameter measurements between laboratories. Associated 95% confidence intervals for r and R were calculated using the delta formula described by Weisberg (2005). To ensure convergence of the estimation, isolate/antimicrobial combinations with a median value less than the 5th percentile (i.e., 8 mm) were excluded from the analysis. In this study, participants were assumed to represent the population of laboratories that routinely perform the disc-diffusion assay, allowing laboratory to be modelled as a random effect. The *E. coli* isolates were not considered representative of the *E. coli* population in pigs and kept as a fixed effect in the model. Independence and normality of the distribution of the residuals were assessed using residual diagnostic plots.

The model used to obtain estimates of variance for each antimicrobial was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \pi_k + \tau_{jk} + \omega_{ik} + \varepsilon_{ijk} \quad (1)$$

where Y_{ijk} is zone-diameter measurement made on the ith isolate in the jth batch by the kth lab, μ is the mean intercept, α_i is the fixed effect of the ith isolate, β_j is the fixed effect of jth batch, $\alpha\beta_{ij}$ is the fixed interaction between isolate and batch, π_k is the random effect of kth laboratory, τ_{jk} is the random interaction between batch j and laboratory k, ω_{ik} is the random interaction between isolate i and laboratory k, and ε_{ijk} is the residual error term.

The equation for calculating the r estimate ($\hat{r}$) and the R estimate ($\hat{R}$) (ISO, 1994), respectively is:

$$\hat{r} = 1.96\sqrt{2\hat{\sigma}^2} \quad (2)$$

$$\hat{R} = 1.96\sqrt{2(\hat{\sigma}^2_\pi + \hat{\sigma}^2_\tau + \hat{\sigma}^2_\omega + \hat{\sigma}^2)} \quad (3)$$

where σ^2 is the variance of the residual error; σ^2_π is the variance due to laboratory; σ^2_τ is the variance due to crossed effects between laboratory and batch; σ^2_ω is the variance due to crossed effects between isolate and laboratory.

Table 1
Epidemiologic Cut-off Values (ECOFF), Clinical Laboratory Standards Institute (CLSI) zone diameter interpretative criteria and ATCC 29522 quality control strain ranges for porcine *Escherichia coli* isolates evaluated in this study.

Antimicrobial	Abbreviation	ECOFF[a] (mm)	CLSI Susceptible breakpoint (mm)	CLSI Resistant breakpoint (mm)	ATCC 29522[b] range (mm)
Ampicillin	AMP	≥14	≥17*	≤13*	15–22
Ceftiofur	CFT	NA	≥21†	≤17†	26–31
Chloramphenicol	CHL	≥17	≥18*	≤12*	21–27
Gentamicin	GEN	≥16	≥16†	≤12†	19–26
Tetracycline	TET	NA	≥15*	≤11*	18–25
Trimethoprim-Sulfamethoxazole	SXT	≥16	≥16*	≤10*	23–29

[a] ECOFFs derived from EUCAST disc diffusion database (https://mic.eucast.org/Eucast2/).
[b] CLSI VET08: Table 4A. Disc Diffusion QC Ranges for Nonfastidious Organisms.
* CLSI M100-S25. Table 2A. Zone diameter and minimum inhibitory concentration interpretative criteria for *Enterobacteriaceae*.
† CLSI VET08: Table 2A. Zone diameter and minimum inhibitory concentration interpretative criteria for *Enterobacteriaceae*.

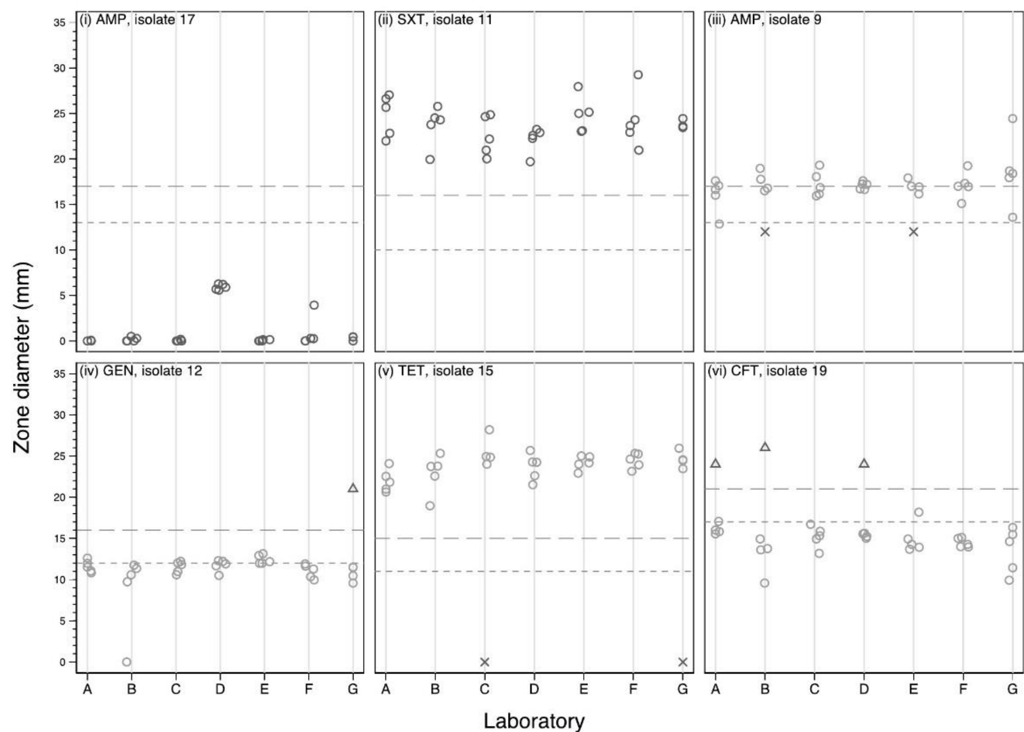

Fig. 1. Variation in disc diffusion zone-diameter (mm) measurements for selected combinations of porcine *Escherichia coli* isolates and antimicrobials obtained from seven veterinary laboratories (A–G). Disc diffusion measurements were reported five times per laboratory. SXT (trimethoprim sulfamethoxazole), AMP (ampicillin), TET (tetracycline), CFT (ceftiofur) and GEN (gentamicin). Horizontal long-dash lines represent the Clinical and Laboratory Standards Institute (CLSI) susceptible breakpoints and horizontal short-dash lines represent resistant breakpoints for each antimicrobial. Triangle symbol represents a very major error (replicate classified as susceptible when the actual status is resistant). Cross symbol represents a major error (replicate classified as resistant when the actual status is suceptible). Other combinations of isolate and drug are given in Supplementary materials.

3. Results

3.1. Descriptive results

In total, the seven participating laboratories generated 4200 zone-diameter measurements on 20 distinct *E. coli* isolates across six antimicrobial agents in a completely balanced dataset (no missing values). All laboratories participating in this study measured the zone-diameter manually using a ruler or calipers.

Plots of repeated zone-diameter measurements for a selected number of *E. coli*/antimicrobial combinations are shown in Fig. 1 (a full set of plots for each combination of isolate and antimicrobial agent are available in Supplementary Materials). In general, the variation in zone-diameter measurements for resistant isolates was small (Fig. 1(i)), while variation in measurements for susceptible isolates was much higher (Fig. 1(ii)). While variation in repeated measurements at the extremes of the measurement scale (i.e., < 10 mm or > 20 mm) rarely affected clinical interpretation, there was a heightened risk of misclassification when such variation approximates the breakpoints as illustrated in Fig. 1(iii) and (iv). Major errors (false-resistance) was evident in Fig. 1(v), while very major errors (false-susceptible) was evident in Fig. 1(vi). Fig. 1(i) reveals major inconsistencies in reporting of complete inhibition of bacterial growth, with only one laboratory correctly recording complete inhibition (6 mm, the diameter of antimicrobial discs) according to CLSI protocols. For ceftiofur, where resistance is rare in porcine *E. coli* isolates in Australia (thus requiring purposeful inclusion of isolates obtained by Abraham et al. (2018)), laboratories were consistent at reporting full susceptibility (Supplementary Fig. 2), however, some misclassification errors were present when determining resistance (Fig. 1(vi)). When the analysis was restricted to the ATCC 25922 quality control strain, only trimethoprim-sulfamethoxazole had all repeated measurements (n = 35, median 27 mm) within the reference range, while only nine ceftiofur measurements fit within the reference range (median 25 mm) (Fig. 2).

Descriptive statistics relating to the central measures of tendency are available in Supplementary Table 2. The occurrence of misclassifications, where individual zone-diameters were compared to the overall mean interpretation per isolate/antimicrobial combination, are reported in Table 2. The observed very major error rate (1.62%) was marginally higher than the very major error rate of < 1.5% specified in ISO 20776-2 (2006), while the major error rate (1.58%) was within the ISO acceptable error level of < 3%. The very major error rates reported for ceftiofur (3.81%) and trimethoprim-sulfamethoxazole (2.86%) were higher than the ISO acceptable error level, as was the major error rate for ampicillin (6.86%). For ampicillin, many zone-diameter measurements clustered between 16–18 mm, resulting in a large minor error rate (8.43%). Where ECOFFs were available for comparison to clinical breakpoints, the overall very major error rate (1.22%) was within the ISO 20776-2 acceptable error rate. However, the major error rate (4.78%) was well outside of the acceptable error rate (Supplementary Table 3).

While recording 0 mm instead of 6 mm when there was no inhibition zone does not affect clinical interpretation, it does affect estimation methods used to evaluate the assay's precision. Zone-diameter

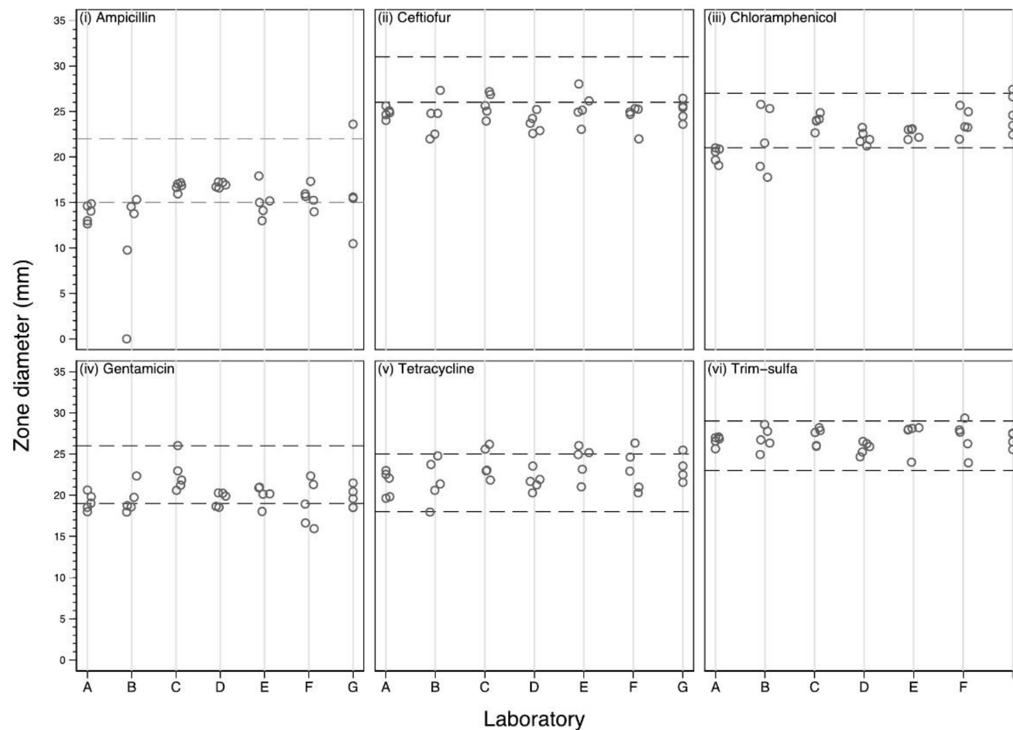

Fig. 2. Variation in disc diffusion zone-diameter measurements for the ATCC 25922 *Escherichia coli* quality control strain for six antimicrobial agents obtained from seven laboratories (A–G). Disc diffusion measurements were reported five times per laboratory. Horizontal dashed lines represent the Clinical and Laboratory Standards Institute (CLSI) quality assurance reference range for ATCC 25922.

measurements < 6 mm were therefore corrected to 6 mm. For each antimicrobial, the range of within-isolate coefficients of variation (CV) values is reported in Fig. 3 and Supplementary Table 4. Isolates outside of the inter-quartile ranges also recorded the highest rates of misclassification errors (Fig. 3). Ampicillin had the most measurement relative variation (median, 11.6%), while had the least trimethoprim-sulfamethoxazole (median CV, 5.8%).

3.2. Repeatability and reproducibility

The estimates $\hat{r}$ and $\hat{R}$ for disc diffusion are reported in Table 3. Overall, $\hat{r}$ across all antimicrobials ranged between 4.4 mm (tetracycline) and 6.6 mm (trimethoprim-sulfamethoxazole), while the $\hat{R}$ were always larger than $\hat{r}$ (5.4 mm, gentamicin and 7.2, trimethoprim-sulfamethoxazole). For interpretation, the expected difference between any two zone-diameter measurements within the same laboratory under the same conditions for ampicillin will rarely (< 5%) exceed

Table 2
Performance of the disc diffusion assay for an intra- and inter-laboratory agreement study where the median zone diameter value was used to define the "actual" susceptibility status to each antibiotic. Susceptible (S), intermediate (I) or resistant (R) status of each replicate was obtained by categorising zone diameter values (mm) using standard interpretative criteria (see footnotes). Errors of different severity (minor, major, very major) occur when an individual replicate's interpretation differs from the interpretation based on the median zone diameter for other replicates of that isolate.

Antibiotic	Total no. of isolate/ antimicrobial combinations	No. of replicates observed as S/I/R	No. of minor errors[a]	No. of major errors[a]	No. of very major errors[a]
Ampicillin*	700	175/70/455	59 (8.43%)	12 (6.86%)	3 (0.66%)
Ceftiofur†	700	595/0/105	23 (3.87%)	3 (0.51%)	4 (3.81%)
Chloramphenicol*	700	350/0/350	11 (3.14%)	3 (0.86%)	5 (1.43%)
Gentamicin†	700	420/0/280	22 (3.14%)	4 (0.95%)	5 (1.79%)
Tetracycline*	700	210/0/490	0 (0.0%)	3 (1.43%)	5 (1.02%)
Trimethoprim-Sulfamethoxazole*	700	280/0/420	7 (1.00%)	7 (2.50%)	12 (2.86%)
Total	4200	2030/70/2100	102 (2.43%)	32 (1.58%)	34 (1.62%)

[a] Error rates are reported according to ISO 20776–2. Minor errors are replicates identified as susceptible or resistant when the actual status is intermediate or vice versa. Major errors are replicates classified as resistant when the actual status is suceptible. Very major errors are replicates classified as susceptible when the actual status is resistant.

* Zone diameter interpretative criteria derived from CLSI M100-S25 (2015), Table 2A.

† Zone diameter interpretative criteria derived from CLSI VET08 (2018), Table 2A.

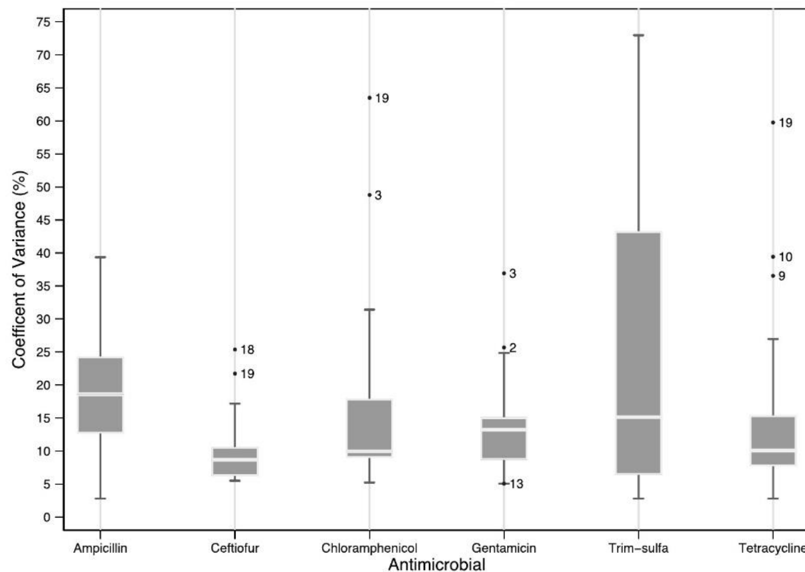

Fig. 3. Boxplot with medians, quartiles, and ranges illustrating the distribution of the within-isolate Coefficient of Variation (CV) of the disc diffusion assay for each assessed antimicrobial. For a given antimicrobial, zone-diameter of an isolate was measured on five occasions by seven laboratories. The boxes denote the interquartile range, the whiskers represent the entire range of CV values observed, and the white lines indicate medians. Numbers outside of the whiskers represent isolates with CV estimates outside of min-max values for each antimicrobial.

Table 3
Estimates of the repeatability (intra-laboratory) and reproducibility (inter-laboratory) coefficients of the disc diffusion assay for six antimicrobials using 20 porcine pathogenic *Escherichia coli* isolates submitted to seven veterinary laboratories on five occasions.

Antimicrobial	Isolate identification number[a]	n[b]	Repeatability[c] (95% CI)	Reproducibility[d] (95% CI)
Ampicillin	1,5,6,9,12,13,20	245	4.6 (4.16, 5.15)	6.5 (4.33, 9.80)
Ceftiofur	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,19,20	665	4.9 (4.59, 5.20)	5.8 (4.22, 7.89)
Chloramphenicol	1,5,6,8,9,10,11,16,17,20	350	5.2 (4.79, 5.70)	6.3 (4.47, 8.80)
Gentamicin	1,2,5,6,7,9,10,11,12,13,14,15,17,19,20	525	4.9 (4.53, 5.21)	5.4 (3.98, 7.23)
Tetracycline	6,11,12,16,20	175	4.4 (3.83, 4.97)	5.6 (3.51, 8.83)
Trimethoprim-sulfamethoxazole	1,6,9,10,11,14,20	245	6.6 (5.93, 7.35)	7.2 (4.47, 11.68)

[a] Isolates were included in the estimation if the minimum measurement for a given isolate/ antimicrobial combination was greater than the 5th percentile (8 mm) of all measurements combined for that antimicrobial agent.
[b] Count of zone diameter measurements analysed for a given antimicrobial agent.
[c] Repeatability is defined as the upper 95% probability bound of the expected difference between any two measurements within the same laboratory.
[d] Reproducibility is defined as the upper 95% probability bound of the expected difference between two any measurements from different laboratories.

4.6 mm, while between laboratories the difference between two zone-diameter measurements for ampicillin will rarely (< 5%) exceed 6.5 mm. The variation reported in disc diffusion measurements both within- and between laboratories for the six antimicrobials was comparable to the reference ranges published in CLSI (2018) for the *E. coli* quality control strain, ATCC 25922. For instance, both $\hat{r}$ (4.6 mm) and $\hat{R}$ (6.5 mm) for ampicillin were within the acceptable ampicillin reference range for ATCC 25922 (7 mm). Variance components estimates from the linear mixed model used to calculate $\hat{r}$ and $\hat{R}$ are reported in Table 4. The contribution towards the total variance was broken down across the sources of error in the data, of importance is the crossed effects between laboratory and batch (range 11.4%–32.8%), and the error term (range 50.4%–83.6%). Batch to batch variation was higher within some laboratories.

4. Discussion

Surveillance of antimicrobial resistance in a wide range of veterinary pathogens and animal species is achievable if susceptibility data can be collected from veterinary laboratories that routinely test bacterial isolates. Use of this data in national surveillance efforts will be greatly enhanced if there is a strong understanding of the performance of the assay used, and confidence in the competency of the laboratories performing the assay. To further our understanding, this study evaluated the precision of the disc diffusion assay to a blinded panel of pathogenic *E. coli* strains for six antimicrobials, involving major veterinary laboratories located in all states of Australia. By evaluating pathogenic *E. coli* strains, we have performed the study in a context which reflects everyday scenarios in veterinary laboratories, thereby providing a strong basis for making inferences about the precision of the disc diffusion assay.

In this study, the overall difference between two zone-diameter measurements within- or between-laboratories is expected to rarely (< 5%) exceed 4.4–7.2 mm depending on the antimicrobial assessed. While repeatability and reproducibility estimates produced here are a product of the interactions between the assay, isolates, and laboratories that participated in this study, these estimates can be generalised to all laboratories. The disc diffusion assay demonstrated a degree of variability for some antimicrobials which may limit its usefulness for the surveillance of pathogenic *E. coli* isolates in animals, particularly for trimethoprim-sulfamethoxazole and isolates with intermediate susceptibility to ampicillin or low-level resistance to ceftiofur. For example, the inter-laboratory agreement of disc diffusion for ceftiofur was estimated to be 5.8 mm (95% CI, 4.2 mm, 7.9 mm, Table 3), indicating that within the broader population of laboratories that routinely perform disc diffusion using CLSI protocols, two zone-diameter

Table 4
Estimates of variance components used to calculate repeatability (intra-laboratory) and reproducibility (inter-laboratory) estimates for the disc diffusion assay precision study where seven veterinary laboratories assessed the susceptibility of 20 porcine *Escherichia coli* isolates to six antimicrobials on five occasions.

Antimicrobial	Source	Variance (95% CI)	% of model variance
Ampicillin	Lab	0.35 (0.25, 4.86)	6.6%
	Lab x Batch	1.74 (0.92, 3.31)	32.8%
	Lab x Isolate	0.54 (0.23, 1.30)	10.2%
	Error	2.67 (2.16, 3.31)	50.4%
Ceftiofur	Lab	0.28 (0.44, 1.73)	6.7%
	Lab x Batch	0.81 (0.43, 1.51)	19.4%
	Lab x Isolate	0.09 (0.01, 0.71)	2.1%
	Error	2.98 (2.64, 3.38)	71.8%
Chloramphenicol	Lab	0.71 (0.18, 2.76)	14.5%
	Lab x Batch	0.56 (0.24, 1.31)	11.4%
	Lab x Isolate	0.23 (0.05, 1.01)	4.71%
	Error	3.41 (2.87, 4.07)	63.4%
Gentamicin	Lab	0.12 (0.01, 1.17)	3.2%
	Lab x Batch	0.42 (0.19, 0.91)	11.7%
	Lab x Isolate	0.12 (0.02, 0.73)	3.3%
	Error	2.96 (2.56, 3.39)	81.9%
Tetracycline	Lab	0.16 (0.00, 5.95)	4.1%
	Lab x Batch	1.01(0.46, 2.20)	26.1%
	Lab x Isolate	0.32 (0.08, 1.25)	8.4%
	Error	2.38 (1.83, 3.09)	61.5%
Trimethoprim-Sulfamethoxazole	Lab	0.15 (0.00, 5.79)	2.2%
	Lab x Batch	0.82 (0.29, 2.33)	12.6%
	Lab x Isolate	0.10 (0.00, 25.0)	1.6%
	Error	5.45 (4.40, 6.75)	83.6%

measurements for ceftiofur on the same *E. coli* isolate would not be expected to differ by more than 5.8 mm, 95% of the time. Measurement variation of 5.8 mm is critical the closer the zone-diameter is to the interpretative criteria where the likelihood of major or very major errors is high. Of note, the repeatability and reproducibility estimates for all six antimicrobials were mostly within the ranges specified for the ATCC 25922 quality control strain (CLSI, 2018), suggesting that the level of variation in zone diameter measurements seen with pathogenic and less understood isolates is tolerable when compared to a stable, well-characterised strain.

The residual error term (range 50.4%–83.6%, Table 4) is the largest factor contributing to the assay's variation for the panel of antimicrobials evaluated. While the factors that contribute to this variation remain unknown, it is likely that some of the variation is due to inconsistencies in the manual measurement of zone-diameters. Knowledge of the extent of laboratory to laboratory variation is valuable as this information can be used to standardise the assay further to minimise measurement error. For instance, the findings presented here add weight to the call to find new ways to consistently measure zone-diameters, such as the adoption of automated zone readers which have been previously reported to eliminate much of the measurement uncertainty associated with manual reading (Lestari et al., 2008; Hombach et al., 2013; Idelevich et al., 2016). Reducing the level of variation in zone-diameter measurements will increase confidence in the assay, particularly for isolates exhibiting decreased susceptibility to important antimicrobials.

When very major, and major error rates are taken into consideration, there is good evidence to demonstrate that the network of laboratories that participated in this study are competent at performing the disc diffusion assay when strains are predictable or at the extremes of sensitivity (i.e., truly susceptible or truly resistant). Other studies that have reported on the reproducibility of disc diffusion or on outcomes from proficiency testing, have also found overall high performance with well-characterised quality control strains (Medeiros and Crellin, 2000; Tenover et al., 2001; Luzzaro et al., 2006; Hegstad et al., 2014; Matuschek et al., 2014; Hombach et al., 2017). However, the very major error rates reported for ceftiofur (3.81%) and trimethoprim-sulfamethoxazole (2.86%), and the major error rate for ampicillin (6.86%) point to some concern regarding the precision of the assay when testing pathogenic strains that may be either (i) biologically unpredictable or have low-level resistance (e.g. ceftiofur); (ii) have zone edges that are challenging to read (e.g. trimethoprim-sulfamethoxazole); or (iii) have intermediate susceptibility (e.g. ampicillin).

Analysing the agreement of repeated measurements by categorising the data into susceptible, intermediate or resistant is forgiving when zone-diameters occur at either end of the scale, but less so when measurements cluster near clinical breakpoints. Here, small variations in zone-diameters will result in the misclassification of isolates. For example, in this dataset, minor errors (susceptible – intermediate/ resistant – intermediate) were seen in isolates with decreased susceptibility to ampicillin, and these single-step misclassifications were an important source of variability in the assay for ampicillin. This level of measurement error in a well-used test is an important consideration when deciding if passively acquired laboratory data is suitable for use in national surveillance, especially when monitoring pathogenic bacteria exhibiting decreased susceptibility to important antimicrobials.

CV estimates are calculated to describe measurement variation for assays with continuous outcomes (Jordan et al., 2012; OIE, 2018c). In this study, CV estimates for truly susceptible isolate/ antimicrobial combinations (such as seen with most isolates to ceftiofur (except Isolates 18, 19)) were < 15%, signalling that the relative variability of disc diffusion was small for truly susceptible isolates. However, other data presented here demonstrate the CV on its own can be misleading for describing imprecision of the disc diffusion assay for pathogenic isolates which may have the following characteristics: (i) small dispersion of zone-diameter values (e.g., all results agree within 1–2 mm) resulting in a zero/ very low CV; or (ii) errors resulting in excessively large CVs (e.g., Isolate 19 with chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole). Given these limitations and the highly variable susceptibility seen in pathogenic bacteria, CV estimates on their own should not be used to draw inferences on the precision of the disc diffusion assay.

Estimates generated in the linear mixed model are a product of the characteristics of the assay, the susceptibility status of the isolates, and the competence of the laboratories that participated in the study. However, the available methodology to assess intra- and inter-laboratory agreement only suits scenarios where there is variability of measurements. Multi-modal measurements, as occurs here means that not

all isolates could be accommodated in the existing models used to assess repeatability and reproducibility. For example, many of the randomly selected isolates were resistant to one or more antimicrobials, resulting in zero or minimal variance and had to be excluded from the final model. For pathogenic isolates with low-level antimicrobial resistance (e.g., Isolates 18 and 19 with ceftiofur) or decreased susceptibility (e.g., Isolate 6 for ampicillin), variation in reagents, such as the volume of media dispensed in agar plates and antibiotic disc potency, and the expertise of the technician performing the assay may impact on the accuracy of measurements around the breakpoint zone. Theoretically, smaller repeatability and reproducibility estimates could have been achieved if only well characterised or truly susceptibility isolates were used in the study.

This study was designed to only quantify variation in the assay caused by laboratories, batches, isolates, and their interactions and did not control for other causes of variation. This means the residual error term cannot be broken down into factors that may have contributed to variation such as differences in manual measurement technique, variation in base media, differing manufacturers of agar plates, antibiotic discs, reagents, and other laboratory related performance. For instance, a study by Hombach et al. (2016) examining technical and biological variations in the disc diffusion assay identified the highest relative contribution to variation came from the operator, specifically during inoculum preparation and plate streaking. Plasmid loss during freezing or transportation may also have been a factor in the variation observed between batches across laboratories and the discrepant results for some isolates (e.g., this may explain which Isolate 19 had so many misclassifications). These factors are expected to be a large part of the variation reported here. However, this study sought to reflect the reality of routine disc diffusion testing for a range of pathogenic *E. coli* in a national network of veterinary laboratories and the results presented here replicate that scenario as closely as possible. Where some studies have sought to describe the minimum precision of the assay; ours has sought to describe the maximum precision. It is also possible that in studies such as this, isolates receive more attention than those seen in routine sampling, and that this may introduce bias in the evaluation of performance. Alternatively, the estimates presented here may be the optimal level of precision attained by veterinary laboratories using the CLSI disc diffusion protocol.

5. Conclusion

Overall, disc diffusion susceptibility data generated in veterinary laboratories can be acquired for use in national surveillance provided the assay is consistently performed according to CLSI standards, and the antimicrobial agents used to evaluate susceptibility can be reliably evaluated. For isolates with true susceptibility or true resistance to an antimicrobial, the precision of the disc diffusion assay was satisfactory. The precision of the assay was less favourable for isolates with intermediate susceptibility or low-level resistance, and for antimicrobials where zone edges can be challenging to determine such as occurs with trimethoprim-sulfamethoxazole. Therefore, when defining which antimicrobials to include in panels for the surveillance of antimicrobial resistance in pathogenic *E. coli*, a critical consideration should be the ability of those antimicrobials to yield reliable zone diameters for the organism.

Ethical approval

None required.

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Declaration of Competing Interest

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Appendix A. Supplementary data

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References

Abraham, S., Jordan, D., Wong, H.S., Johnson, J.R., Toleman, M.A., Wakeham, D.L., Gordon, D.M., Turnidge, J.D., Mollinger, J.L., Gibson, J.S., Trott, D.J., 2015. First detection of extended-spectrum cephalosporin- and fluoroquinolone-resistant Escherichia coli in Australian food-producing animals. J. Glob. Antimicrob. Resist. 3, 273–277.

Abraham, S., Kirkwood, R.N., Laird, T., Saputra, S., Mitchell, T., Singh, M., Linn, B., Abraham, R.J., Pang, S., Gordon, D.M., Trott, D.J., O'Dea, M., 2018. Dissemination and persistence of extended-spectrum cephalosporin-resistance encoding IncI1-blaCTXM-1 plasmid among Escherichia coli in pigs. ISME J. 12, 2352–2362.

Barnhart, H.X., Haber, M.J., Lin, L.I., 2007. An overview on assessing agreement with continuous measurements. J. Biopharm. Stat. 17, 529–569.

Bartlett, J.W., Frost, C., 2008. Reliability, repeatability and reproducibility: analysis of measurement errors in continuous variables. Ultrasound Obstet. Gynecol. 31, 466–475.

CLSI, 2013. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals (VET01-A4), 4th edition. Clinical and Laboratory Standards Institute Wayne, PA.

CLSI, 2015a. Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard (M02-A12), 12th edition. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI, 2015b. Performance standards for antimicrobial susceptibility testing; (M100-S25). In: 25th Informational Supplement. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI, 2018. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals (VET08), 4th edition. Clinical and Laboratory Standards Institute, Wayne, PA.

Dargatz, D.A., Erdman, M.M., Harris, B., 2017. A survey of methods used for antimicrobial susceptibility testing in veterinary diagnostic laboratories in the United States. J. Vet. Diagn. Invest. 29, 669–675.

Efron, B., 1987. Better bootstrap confidence intervals. J. Comput. Graph. Stat. 82, 171–185.

Gerke, O., Vilstrup, M.H., Segtnan, E.A., Halekoh, U., Hoilund-Carlsen, P.F., 2016. How to assess intra- and inter-observer agreement with quantitative PET using variance component analysis: a proposal for standardisation. BMC Med. Imaging 16, 54.

Hegstad, K., Giske, C.G., Haldorsen, B., Matuschek, E., Schonning, K., Leegaard, T.M., Kahlmeter, G., Sundsfjord, A., 2014. Performance of the EUCAST disk diffusion method, the CLSI agar screen method, and the Vitek 2 automated antimicrobial susceptibility testing system for detection of clinical isolates of Enterococci with low- and medium-level VanB-type vancomycin resistance: a multicenter study. J. Clin. Microbiol. 52, 1582–1589.

Hombach, M., Jetter, M., Blochliger, N., Kolesnik-Goldmann, N., Bottger, E.C., 2017. Fully automated disc diffusion for rapid antibiotic susceptibility test results: a proof-of-principle study. J. Antimicrob. Chemother. 72, 1659–1668.

Hombach, M., Ochoa, C., Maurer, F.P., Pfiffner, T., Bottger, E.C., Furrer, R., 2016. Relative contribution of biological variation and technical variables to zone diameter variations of disc diffusion susceptibility testing. J. Antimicrob. Chemother. 71, 141–151.

Hombach, M., Zbinden, R., Bottger, E.C., 2013. Standardisation of disk diffusion results for antibiotic susceptibility testing using the sirscan automated zone reader. BMC Microbiol. 13, 225.

Idelevich, E.A., Becker, K., Schmitz, J., Knaack, D., Peters, G., Kock, R., 2016. Evaluation of an automated system for reading and interpreting disk diffusion antimicrobial susceptibility testing of fastidious bacteria. PLoS One 11, e0159183.

ISO, 1994. Accuracy (Trueness and Precision) of Measurement Methods and Results - Part 1: General Principles and Definitions (5725-1). International Organization for Standardization (ISO), Geneva, Switzerland.

ISO, 2006. Clinical Laboratory Testing and in Vitro Diagnostic Test Systems. Susceptibility Testing of Infectious Agents and Evaluation of Performance of Antimicrobial Susceptibility Test Devices. Part 2: Evaluation of Performance of Antimicrobial Susceptibility Test Devices. International Standard 20776-2. International Organization for Standardization (ISO), Geneva, Switzerland.

Jordan, D., Kirkland, P., Morris, S., Coilparampil, R., 2012. Describing the within laboratory and between laboratory agreement of a serum ELISA in a national

laboratory network. Prev. Vet. Med. 104, 240–248.
Kottner, J., Audigé, L., Brorson, S., Donner, A., Gajewski, B.J., Hróbjartsson, A., Roberts, C., Shoukri, M., Streiner, D.L., 2011. Guidelines for reporting reliability and agreement Studies (GRRAS). J. Clin. Epidemiol. 64, 96–106.
Lehtopolku, M., Kotilainen, P., Puukka, P., Nakari, U.M., Siitonen, A., Eerola, E., Huovinen, P., Hakanen, A.J., 2012. Inaccuracy of the disk diffusion method compared with the agar dilution method for susceptibility testing of Campylobacter spp. J. Clin. Microbiol. 50, 52–56.
Lestari, E.S., Severin, J.A., Filius, P.M.G., Kuntaman, K., Duerink, D.O., Hadi, U., Wahjono, H., Verbrugh, H.A., Study Grp Antimicrobial, R., 2008. Comparison of the accuracy of disk diffusion zone diameters obtained by manual zone measurements to that by automated zone measurements to determine antimicrobial susceptibility. J. Microbiol. Meth. 75, 177–181.
Luzzaro, F., Gesu, G., Endimiani, A., Ortisi, G., Malandrin, S., Pagani, L., Rossolini, G.M., 2006. Performance in detection and reporting beta-lactam resistance phenotypes in Enterobacteriaceae: a nationwide proficiency study in Italian laboratories. Diagn. Microbiol. Infect. Dis. 55, 311–318.
Matuschek, E., Brown, D.F.J., Kahlmeter, G., 2014. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. Clin. Microbiol. Inf. 20, O255–O266.
Medeiros, A.A., Crellin, J., 2000. Evaluation of the Sirscan automated zone reader in a clinical microbiology laboratory. J. Clin. Microbiol. 38, 1688–1693.
Murray, P.R., Zeitinger, J.R., Krogstad, D.J., 1982. Reliability of disc diffusion susceptibility testing. Infect. Cont. Hosp. Epidemol. 3, 230–237.
Nijs, A., Cartuyvels, R., Mewis, A., Peeters, V., Rummens, J.L., Magerman, K., 2003. Comparison and evaluation of Osiris and Sirscan 2000 antimicrobial susceptibility systems in the clinical microbiology laboratory. J. Clin. Microbiol. 41, 3627–3630.
OIE, 2018a. Chapter 1.1.6. Principles and methods of validation of diagnostic assays for infectious diseases. Adopted 2013. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 7th edition. World Organisation for Animal Health (OIE), Paris, France.
OIE, 2018b. Chapter 2.1.1 laboratory methodologies for bacterial antimicrobial susceptibility testing. Adopted 2012. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 7th edition. World Organisation for Animal Health (OIE), Paris, France.
OIE, 2018c. Chapter 3.6.5. Statistical approaches to validation. Adopted May 2014. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 7th edition. World Organisation for Animal Health (OIE), Paris, France.
Tenover, F.C., Mohammed, M.J., Stelling, J., O'Brien, T., Williams, R., 2001. Ability of laboratories to detect emerging antimicrobial resistance: proficiency testing and quality control results from the World Health Organization's external quality assurance system for antimicrobial susceptibility testing. J. Clin. Microbiol. 39, 241–250.
Vaz, S., Falkmer, T., Passmore, A.E., Parsons, R., Andreou, P., 2013. The case for using the repeatability coefficient when calculating test-retest reliability. PLoS One 8.
Weisberg, S., 2005. Applied Linear Regression, third edition. John Wiley & Sons, Hoboken, New Jersey.

Supplementary materials for Chapter 4 can be found in Appendix 3

Chapter 5:

Antimicrobial use and stewardship practices on Australian beef feedlots

Contextual Statement

Management of antimicrobial resistance is aided by the collection of data on the use of antimicrobial agents via questionnaires or other survey methods. Information collected at the farm-level is of most value in quantifying antimicrobial agents used, the purposes of use, and the diseases commonly treated. Farm-level data is invaluable in the formulation of relevant, industry-specific antimicrobial stewardship programs, prescribing guidelines, and other communication tools which help manage antimicrobial resistance in animals. In Chapter 5, the beef feedlot sector was used as a case study to examine the usefulness of a common survey method (i.e., self-administered mailed questionnaire) to obtain information on antimicrobial use at the farm-level. This approach relies heavily on farmer participation and support in collecting data which can be legally, commercially, and socially sensitive. Very often, industry bodies associated with the livestock sector are involved in design of the survey. Hence, a strong collaborative approach between all stakeholders is needed to ensure a sufficiently high response rate to make inferences on the broader population. In this study, beef feedlot operators were asked about their antimicrobial use during the previous twelve months, the purposes of use, and the treatment of common disease syndromes. Responses from the survey were used to develop an antimicrobial stewardship program specific to Australian beef feedlots.

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Principal Author

Name of Principal Author (Candidate)	Skye Badger		
Contribution to the Paper	Input into questionnaire, performed analysis and interpreted data, wrote manuscript, corresponding author		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Designed and implemented questionnaire, provided database of feedlot operators		
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Contribution to the Paper	Supervised work, helped in data interpretation, manuscript evaluation		
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Antimicrobial use and stewardship practices on Australian beef feedlots

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Objective Improving antimicrobial stewardship in the livestock sector requires an understanding of the motivations for antimicrobial use and the quantities consumed. However,detailed information on antimicrobial use in livestock sectors is lacking. This cross-sectional study aimed to better understand antimicrobial use in the beef feedlot sector in Australia.

Design A self-administered questionnaire asking about antimicrobial use and reasons for use was designed and mailed to beef feedlot operators in Australia. Respondents were asked to report the percentage of animals treated, purpose of use, and disease conditions targeted for 26antimicrobial agents.

Results In total, 83 of 517 (16.1%) beef feedlot operators completed the survey. Monensin (61.0%of respondents) and virginiamycin (19.5%of respondents) were the most commonly reported in-feed antimicrobials. In-feed antimicrobial agents were most frequently used by respondents for treatment of gastrointestinal diseases (52.8%). Antimicrobials were used for growth promotion by 42.1% of respondents, with most (85.7%) reporting the use of ionophores(a group of compounds not used in human medicine). Short-acting penicillin(69.1%), short-acting oxytetracycline, and tulathromycin (both 57.3%) werethe most common injectable antimicrobial agents used. Injectable antimicrobials were most frequently used to treat respiratory (72.3%) and musculoskeletal (67.5%) conditions.

Conclusion Overall,the use of antimicrobials was appropriate for the purpose indicated, and there was a strong preference for drugs of low-importance in human medicine. The data described here stand to be a strong influence on the implementation of an antimicrobial stewardship program in the sector.

Keywords antimicrobial; cattle; prudent; resistance; stewardship; veterinary

Abbreviations ASTAG, Australian Strategic and Technical Advisory Group on Antimicrobial Resistance; LA, long-acting; NAHMS, National Animal Health Monitoring System, USA; SA, short-acting

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An important global strategy for the control of antimicrobial resistance is the implementation of antimicrobial stewardship programs in human and animal healthcare settings. As such, all uses of antimicrobials, including those involving food animals, are being scrutinised to identify opportunities to reduce selection pressure in bacterial populations.[1–3] The development of antimicrobial stewardship programs in food animals requires a solid understanding of the necessity and quantity of antimicrobials used in differing livestock sectors. However, in many countries, information on antimicrobial usage in food animals is not readily available, and so the quantity and types of antimicrobials used in each livestock sector are largely unknown.[4] For instance, in Australia, antimicrobial usage data in animals are limited to nationally aggregated data that define the weight of active constituents sold for each animal species with little other accompanying information.[5] To respond to community expectations for the judicious use of antimicrobials, more detailed data are required at the herd level, where key decisions on antimicrobial use are made. This information not only has the potential to stimulate improvement in antimicrobial stewardship among veterinarians and animal owners but will also benefit to those who regulate antimicrobial use in food animals.

The Australian Strategic and Technical Advisory Group on Antimicrobial Resistance (ASTAG) assigns ratings to antimicrobials according to their importance to human health.[6] The medical and veterinary sectors use these ratings as a guide for the registration of antimicrobial agents,[7] the interpretation of usage practices, and for the development of prescribing guidelines and antimicrobial stewardship programs. The primary aim of veterinary antimicrobial stewardship is to minimise the development of bacterial resistance to antimicrobials of importance to human health while maintaining the efficacy of antimicrobials for use in diseased animals.[8,9] Judicious use of antimicrobials ideally limits their administration to clinically infected individuals rather than groups comprised of healthy and diseased animals. However, in some production systems, antimicrobials are administered to groups of animals for a range of reasons including treatment of the clinically diseased, prevention of infection and growth promotion (increased feed efficiency and daily weight gain).

High standards of antimicrobial stewardship in the beef feedlot sector are necessary, given its growing importance to the Australian economy. In 2017–2018, 2.8 million grain-fed cattle were marketed in Australia, representing 38% of all adult cattle slaughtered.[10] In the same period, Australian grain-fed beef products were exported to major international markets, including Japan, South Korea, China, the European Union and the United States.[11] Growth in beef feedlot production globally and increased interest in the impact of antimicrobial resistance on animal health and public health has led to a greater focus on antimicrobial use in this sector.[12–14]

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Bovine respiratory disease is considered the most significant cause of morbidity and mortality in feedlot cattle and the greatest motivation for the use of antimicrobials.[12,15] Ruminal acidosis and lameness are also common conditions requiring therapy.[12] However, the contribution of these diseases to antimicrobial use in feedlots has not been well quantified. Indeed, there is scant published information available on antimicrobial uses and practices in the beef feedlot sector of any of the major cattle-producing countries of the world. The exception is the United States, which periodically publishes the National Animal Health Monitoring System (NAHMS) survey of antimicrobial use in cattle feedlots.[16–18] The NAHMS survey captures data on the types of antimicrobials used in US feedlots, administration route (injection, in-feed and in-water), treatment of common infectious diseases and antimicrobial stewardship practices. Information generated by surveys such as NAHMS are necessary to inform country-specific policies that optimise antimicrobial use in feedlot cattle.

Therefore, this study aimed to investigate antimicrobials used in Australian beef feedlots over the previous 12 months, by route of administration (injectable or in-feed), the purpose of treatment (individual treatment, group treatments) and disease syndromes, and also to describe management practices associated with the treatment of animals. The study focuses on a subset of data yielded by a more comprehensive questionnaire of antimicrobial use, antimicrobial stewardship and quality assurance practices. The data sought here are a key step in the development of antimicrobial stewardship guidelines for beef feedlots, and the design of future studies investigating factors that influence antimicrobial use in the sector.

Materials and methods

Study population

The target population was beef feedlots operating in Australia in 2017. The criteria for inclusion as a feedlot were based on the Australian Lot Feeders Association definition of a 'constructed facility with designated water points where cattle are confined with a stocking density of 25 m^2 per standard cattle unit or less and are only fed a prepared ration for the purposes of production'.[19] The source population comprised a composite list of 517 eligible beef feedlots in Australia derived from commercially sensitive sources and the client databases of five veterinary feedlot consultants who conducted the survey through their practices. Feedlots included in the source population were located in all mainland States and Territories of Australia except the Australian Capital Territory, with 264 feedlots from Queensland, 118 from New South Wales, 62 from Victoria, 36 from South Australia, 35 from Western Australia and one feedlot each from Tasmania and the Northern Territory. The final study group comprised those eligible beef feedlot operators who responded to the survey. Feedlot operators were encouraged to participate via an information pack provided by the Australian Lot Feeders Association and Meat and Livestock Australia. The information pack included an introductory letter from Meat and Livestock Australia (the industry research and development body), fact sheets on antimicrobial use in the cattle industry and the questionnaire. Other than the author (K.F. Sullivan) who compiled the list of eligible feedlots and supervised data entry of returned questionnaires, all other authors involved in the study were blinded to the identity of the respondents. K.F. Sullivan was not involved in the statistical analysis of responses.

Questionnaire

The questionnaire (Data S1) was designed to capture information on the antimicrobial agents used and the reasons for use in cattle held in the feedlot in the previous 12-month period. Additional questions were asked of the involvement of veterinarians in the determination of antimicrobial use, and the storage and stock auditing of antimicrobials and over-the-counter veterinary chemicals. The questionnaire consisted of 98 questions grouped into five sections:

Section 1: General feedlot information. This section asked for background information including the holding capacity of the feedlot, the number of animals sold per annum, average days on feed and the percentage of total animals that required treatment in a hospital pen in the previous 12-month period.

Section 2: Antimicrobial use. This section asked separately for the injectable and in-feed use of 26 antimicrobials in the previous 12 months. The selected antimicrobials covered all antimicrobial classes and comprised drugs rated by ASTAG as low, medium and high importance to human health. When an antimicrobial was used, the respondent was asked to estimate the proportion of animals treated (within the previous 12 months) and to select at least one of the following purposes of use: 'individual animal' treatment, 'mass' treatment (i.e. in response to a disease outbreak), 'timed' treatment (i.e. the timed/scheduled short-term treatment of animal lots), prevention (i.e. long-term treatment of animal lots to prevent disease), or growth promotion (i.e. use of an antimicrobial to improve physiological performance). When an antimicrobial was used therapeutically, the respondent was asked to select at least one of the following disease syndrome/s: respiratory, digestive, musculoskeletal, neurological, urogenital or 'other diseases'.

Section 3: Veterinary treatment protocols. This section included questions on the frequency of veterinary visits in the previous 12-month period; the existence of standard protocols for the management of newly introduced animals, and the treatment of animals using antimicrobial agents; whether these protocols were followed by feedlot staff; and if feedlot staff assessed animals for response to treatments prior to returning to their home pen.

Section 4: Supply and use of veterinary chemicals. This section of the questionnaire included a series of questions related to the supply and purchase of prescription and over-the-counter animal health products, access and administration of veterinary chemicals, identification of treated animals and training in the administration of veterinary chemicals.

Section 5: Storage and chemical stock control. This section included questions on the storage and auditing of veterinary chemicals.

All questions were closed-ended, multiple-choice questions, except for questions where 'other' could be selected. Here an open text field was available for the respondent to elaborate. For all questions, the response options of 'do not know' and 'unanswered' were available.

The questionnaire was pilot tested by telephone interview with five feedlot operators. Each pilot test took between 20 to 25 minutes to

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complete. While it was initially planned to administer the questionnaire by telephone, following a review of the pilot-testing process a decision was made to change to a mailed questionnaire. This was to allow respondents more time to complete the questionnaire and consult herd records where necessary. Minor amendments were made to adapt the questionnaire to being paper-based (comprising 14 pages). The questionnaire was mailed to the 517 eligible beef feedlot operators in February 2017. Feedlots who did not respond within three to 4 weeks were re-contacted by email with an electronic version of the questionnaire included. The questionnaire was designed using online software (Qualtrics, https://www.qualtrics.com/au/) and data from the returned questionnaires were entered manually into the online form. One respondent was contacted by K.F. Sullivan to clarify a confusing response.

Statistical analysis

For this study, analysis and reporting of survey results were restricted to the first three sections of the questionnaire.

Data were transferred from the survey software platform as comma-separated values file into Stata version 15.1 (Stata Corporation, College Station, TX, USA) for analysis. Descriptive statistics were calculated using unadjusted frequency counts, with proportions reported as the number of respondents selecting an answer-option divided by the total number of respondents attempting the question. Responses of 'do not know' and 'unanswered' were interpreted as missing values (not included in the analysis). Some response categories were merged when redundant or to improve interpretation. Frequency of response categories was compared across feedlot capacity using the Fishers' exact test with associated two-sided P-values for interpretation at the 5% level of significance.

Results

Profile of respondents

The survey period extended from 13 February to 1 July 2017, with 16.1% (83/517) of questionnaires returned. Of responses received, 48 came from Queensland, 23 from New South Wales, six from Victoria, and three from South Australia and Western Australia, respectively (Table 1). There was no statistical difference between the source population and respondents across the State categories ($P = 0.48$). Feedlots were categorised by their capacity, with most respondents indicating fewer than 3000 cattle ($n = 46$). Feedlots were further categorised by the number of cattle sold, number of days on feed and proportion of cattle requiring treatment in the previous 12 months. For most respondents ($n = 46$), less than 10,000 cattle were sold, the average number of days on feed was between 80 and 150 days ($n = 50$), and 10% or fewer cattle were removed for treatment in hospital pens ($n = 58$) (Table 1).

Table 1. Characteristics of respondents to a survey of antimicrobial use and practices in Australian beef feedlots over 12 months

Respondent profile	Source population	Frequency (%)
		Respondents
Geographic location by state or territory		
Queensland	264	48 (9.3)
New South Wales	118	23 (4.4)
Victoria	62	6 (1.2)
South Australia	36	3 (0.6)
Western Australia	35	3 (0.6)
Northern Territory	1	0 (0.0)
Tasmania	1	0 (0.0)
Total	517	83 (16.1)
Feedlot capacity		
<3000 cattle		46 (55.4)
3000–10,000 cattle		13 (15.7)
>10,000 cattle		23 (27.7)
Unanswered		1 (1.2)
Total		83 (100.0)
Number of cattle sold		
<10,000		46 (55.4)
10,000–20,000		9 (10.8)
20,001–30,000		8 (9.6)
30,001–40,000		4 (4.8)
>40,000		14 (16.9)
Unanswered		2 (2.4)
Total		83 (100.0)
Average number of days cattle kept on feed		
<80 days		27 (32.5)
80–15 days		50 (60.2)
>150 days		6 (7.2)
Unanswered		0 (0.0)
Total		83 (100.0)
Proportion of cattle requiring treatment in hospital pen		
Nil		12 (14.5)
0.1%–2%		6 (7.2)
2.1%–10%		40 (48.2)
10.1%–20%		7 (8.4)
>20%		7 (8.4)
Unanswered		11 (13.3)
Total		83 (100.0)

Frequency and purpose of antimicrobial use

In the 12 months before the survey, the most common injectable antimicrobials used in the feedlots of respondents were short-acting (SA) penicillin (69.1%), followed by SA oxytetracycline and tulathromycin (both 57.3%) (Table 2). SA and long-acting (LA) formulations of ceftiofur were used by approximately one-third of feedlots (35.4% and 34.1%, respectively). The most frequently used in-feed antimicrobial was monensin (61.0%), belonging to the ionophore class which is not used in human medicine, followed by virginiamycin (19.5%), a streptogramin, considered of high importance to humans in Australia (Table 2). For most respondents, fewer than 10% of cattle pulled for treatment were given any injectable

Table 2. Proportion of feedlot respondents that treated cattle with any injectable or in-feed antimicrobials in the 12 months prior to the survey of Australian beef feedlots

Antimicrobial[a]	Antimicrobial class	ASTAG importance ranking[b]	n[c]	Frequency	% use (95% CI)
Injectable					
SA Ceftiofur	Beta-lactam	High	82	29	35.4 (25.1 to 46.7)
LA Ceftiofur	Beta-lactam	High	82	28	34.1 (24.0 to 45.4)
Trimethoprim-sulphonamides	Folic acid inhibitor, sulphonamide	Medium	82	21	25.6 (16.6 to 36.4)
Tylosin	Macrolide	Low	81	4	4.9 (1.4 to 12.2)
Tilmicosin	Macrolide	Low	81	27	33.3 (23.2 to 44.7)
Erythromycin	Macrolide	Low	81	2	2.5 (0.3 to 8.6)
Tulathromycin	Macrolide	Low	82	47	57.3 (45.9 to 68.2)
Florfenicol	Phenicol	Low	82	3	3.7 (0.8 to 10.3)
SA Oxytetracycline	Tetracycline	Low	82	47	57.3 (45.9 to 68.2)
LA Oxytetracycline	Tetracycline	Low	82	17	20.7 (12.6 to 31.1)
SA penicillin	Beta-lactam	Low	81	56	69.1 (57.9 to 78.9)
LA penicillin	Beta-lactam	Low	82	31	37.8 (27.3 to 49.2)
Amoxicillin	Beta-lactam	Low	81	7	8.6 (3.5 to 17.0)
In-feed					
Virginiamycin	Streptogramin	High	82	16	19.5 (11.6 to 29.7)
Tylosin	Macrolide	Low	82	3	3.7 (0.8 to 10.3)
Oxytet/ chlortetracycline	Tetracycline	Low	81	12	14.8 (7.9 to 24.4)
Monensin	Ionophore	No-human use	82	50	61.0 (49.6 to 71.6)
Lasalocid	Ionophore	No-human use	82	5	6.1 (2.0 to 13.7)
Flavophospholipol	Glycophospholipid	No-human use	79	10	12.7 (6.2 to 22.0)

[a]There was no reported use of injectable neomycin, gentamicin and enrofloxacin or in-feed tilmicosin, trimethoprim-sulphonamide, salinomycin and narasin. [b]ASTAG antimicrobial importance ratings. [c]Count of respondents. ASTAG, Australian Strategic and Technical Advisory Group on Antimicrobial Resistance; CI, confidence interval; LA, long-acting; SA, short-acting.

antimicrobial agent (Table 3). Of those respondents that indicated any use of SA ceftiofur (35.2%), almost all used it in less than 2% of cattle pulled for treatment. Of those feedlots indicating any use of LA ceftiofur (31%), most used the drug in less than 2% of cattle pulled for treatment (Table 3). Thirty respondents estimated 10% or more pens were given any in-feed antimicrobial, with the non-medically important antimicrobial ionophores most frequently given (n = 17). Four respondents indicated the use of in-feed virginiamycin in 10%–20% of pens in the previous 12 months (Table 3).

Categories of feedlot capacity were merged to facilitate comparison of antimicrobial use between feedlots. Two categories were created – small feedlots with <3000 cattle and large feedlots with ≥3000 cattle (Table 4). The proportion of feedlots using most injectable antimicrobials was significantly higher in large feedlots compared to small feedlots ($P < 0.05$) (Table 4) except for LA penicillin ($P = 0.49$) and LA oxytetracycline which had marginal non-significant ($P = 0.06$). For low use injectable antimicrobials such as florfenicol, tylosin and erythromycin, there was no significant difference between use given feedlot size ($P > 0.05$). For in-feed antimicrobials, significant differences between large and small feedlot categories were demonstrated for virginiamycin, oxytetracycline/chlortetracycline, monensin and flavophospholipol ($P < 0.01$). See Table S1, Supporting Information for detail on antimicrobial use by feedlot capacity.

Overall, respondents most frequently used injectable antimicrobials to treat individual animals and less frequently for the treatment of animal groups (mass treatment or timed/ scheduled treatment) (Table 5). When treating individual animals, low-importance antimicrobial classes were most widely used such as the SA and LA formulations of tetracyclines and penicillins. Of those feedlots indicating the use of SA or LA ceftiofur, the overwhelming majority restricted use to individual animal treatments, except for three feedlots which indicated use for timed treatments. In-feed antimicrobials were most commonly used for preventative treatments compared to mass treatment, timed treatment or growth promotion (Table 6). Of the feedlots that indicated the use of antimicrobials for growth promotion (n = 35), most (85.7%) used ionophore or phosphoglycolipid classes of antimicrobial agents. Five feedlots reported the use of virginiamycin for growth promotion (Table 6).

Injectable antimicrobials were frequently used to treat respiratory disease (88.2%), musculoskeletal conditions (63.6%) and urogenital diseases (51.5%) (Table 7). The most common injectable antimicrobials used for respiratory disease were tulathromycin (71.7%), SA oxytetracycline (63.3%) and LA ceftiofur (45.0%). For musculoskeletal conditions, SA penicillin (82.1%), followed by LA penicillin (48.2%) were the most common injectable antimicrobials. Of those respondents that used SA or LA ceftiofur, the majority used the drug

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Table 3. Proportion of feedlot respondents that treated cattle with any injectable or in-feed antimicrobials, by antimicrobial and the percent of animals pulled for treatment with an injectable antimicrobial or percent of lots given in-feed antimicrobials in the previous 12 months

Antimicrobial[a]	ASTAG importance ranking[b]	n[c]	Frequency (%) of feedlots in each category				
			0%[d]	<2%	2%–10%	10%–20%	>20%
			Percent of animals pulled for treatment receiving an antimicrobial by injection				
Injectable							
SA ceftiofur	High	71	46 (64.8)	23 (32.4)	2 (2.8)	0 (0)	0 (0)
LA ceftiofur	High	71	49 (69.0)	17 (23.9)	4 (5.6)	1 (1.4)	0 (0)
Trimethoprim-sulphonamides	Medium	71	54 (76.1)	15 (21.1)	2 (2.8)	0 (0)	0 (0)
Florfenicol	Low	71	68 (95.8)	3 (4.2)	0 (0)	0 (0)	0 (0)
Tylosin	Low	70	66 (94.3)	4 (5.7)	0 (0)	0 (0)	0 (0)
Tilmicosin	Low	70	48 (68.6)	16 (22.2)	4 (5.7)	1 (1.4)	1 (1.4)
Erythromycin	Low	71	69 (97.2)	2 (2.8)	0 (0)	0 (0)	0 (0)
Tulathromycin	Low	72	33 (45.8)	20 (27.8)	17 (23.6)	2 (2.8)	0 (0)
SA oxytetracycline	Low	71	29 (40.8)	28 (39.4)	13 (18.3)	0 (0)	1 (1.4)
LA oxytetracycline	Low	71	60 (84.5)	10 (14.1)	1 (1.4)	0 (0)	0 (0)
SA penicillin	Low	70	26 (37.1)	29 (41.4)	11 (15.7)	3 (4.3)	1 (1.4)
LA penicillin	Low	71	45 (63.4)	24 (33.8)	1 (1.4)	0 (0)	1(1.4)
Amoxicillin	Low	71	64 (90.1)	4 (5.6)	2 (2.8)	1 (1.4)	0 (0)
			Percent of lots (pens) given in-feed antimicrobials				
In-feed							
Virginiamycin	High	71	58 (81.7)	4 (5.6)	5 (7.0)	4 (5.6)	0 (0)
Tylosin	Low	70	66 (94.2)	4 (5.7)	0 (0)	0 (0)	0 (0)
Oxytet/chlortetracycline	Low	70	61 (87.1)	4 (5.7)	2 (2.9)	2 (2.9)	1 (1.4)
Monensin	No-human use	68	29 (42.6)	2 (2.9)	21 (30.9)	8 (11.8)	8 (11.8)
Lasalocid	No-human use	70	66 (94.2)	3 (4.3)	0 (0)	0 (0)	1 (1.4)
Flavophospholipol	No-human use	68	58 (85.3)	2 (2.9)	2 (2.9)	3 (4.4)	3 (4.4)

[a]There was no reported use of neomycin, gentamicin, enrofloxacin, in-feed tilmicosin, trimethoprim-sulphonamide, salinomycin and narasin. [b]ASTAG antimicrobial importance ratings for humans. [c]Count of respondents. [d]Respondents that indicated they did not use the antimicrobial. Excludes respondents who selected 'do not know' or 'unanswered'. ASTAG, Australian Strategic and Technical Advisory Group on Antimicrobial Resistance; LA, long-acting; SA, short-acting.

for respiratory disease (85.7% and 96.4%, respectively). Some feedlots also reported use of SA and/or LA ceftiofur for musculoskeletal (21.4% and 10.7%, respectively), gastrointestinal (3.6% and 7.1%, respectively), neurological (3.6% and 10.7%, respectively) and urogenital (7.1% and 3.6%, respectively) conditions. In-feed antimicrobials were most commonly used for gastrointestinal diseases (52.8%), with monensin being the preferred in-feed antimicrobial (96.4%) (Table 7). Of those feedlots that used virginiamycin, the control of gastrointestinal diseases was the most common use (56.3%), although feedlots also reported using virginiamycin for other syndromes, including respiratory (n = 3), musculoskeletal (n = 2), neurological (n = 2), urogenital (n = 1) and 'other' (n = 2) (Table 7).

Veterinary interaction and protocols

Antimicrobial stewardship begins with the interaction between the veterinarian and feedlot operator, where decisions are made about the most appropriate antimicrobials to treat and prevent disease on feedlots. Of the 83 respondents to this questionnaire, most (83.1%) indicated that a veterinarian visited the feedlot premises on a least one occasion in the previous 12 months. Of which, 24 respondents (28.9%) indicated a veterinarian visited at least monthly. Most respondents (57.8%) indicated the feedlot had a veterinary treatment protocol/ prescribed veterinary medicines list (issued by a veterinarian to guide the treatment of sick animals). When respondents were categorised into large capacity feedlots (≥3000 cattle) and small capacity feedlots (<3000 cattle), 88.9% (32/36) of respondents with large feedlots reported the use of a veterinary treatment protocol compared to 35.6% (16/45) of small capacity feedlots ($P < 0.0001$). Almost all feedlots (92.8%) had a documented induction protocol for handling new arrivals. 89.0% of respondents assessed animals for a response to treatment before returning animals to their home pen.

Discussion

While the low response rate (16.1%) to this survey does not allow for strong extrapolation of the findings to the broader population of Australian beef feedlots, the information presented here is an important first step in understanding antimicrobial use and antimicrobial

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Table 4. Proportion of feedlot respondents that treated cattle with any injectable or in-feed antimicrobials, by antimicrobial given and by feedlot capacity (number of cattle held) in the previous 12 months

Antimicrobial[a]	ASTAG importance ranking[b]	n[c]	Feedlot capacity			
			All feedlots (%)	Small (<3000) (%) (user/respondents)	Large (≥3000) (%) (user/respondents)	P-value
Injectable						
SA Ceftiofur	High	81	29 (35.8)	22.2 (10/45)	52.8 (19/36)	<0.01
LA Ceftiofur	High	81	28 (34.6)	22.2 (10/45)	50.0 (18/36)	0.01
Trimethoprim-sulphonamides	Medium	81	21 (25.9)	11.1 (5/45)	44.4 (16/36)	<0.01
Florfenicol	Low	81	3 (3.7)	2.2 (1/45)	5.6 (2/36)	0.58
Tylosin	Low	80	4 (5.0)	2.2 (1/44)	8.3 (3/36)	0.32
Tilmicosin	Low	80	27 (33.6)	17.8 (8/45)	54.3 (19/35)	<0.01
Erythromycin	Low	81	2 (2.5)	2.2 (1/45)	2.8 (1/36)	1.0
Tulathromycin	Low	81	46 (56.8)	33.3 (15/45)	86.1 (31/35)	<0.01
SA oxytetracycline	Low	81	47 (58.0)	40.0 (18/45)	80.6 (29/36)	<0.01
LA oxytetracycline	Low	81	17 (21.0)	28.9 (13/45)	11.1 (4/36)	0.06
SA penicillin	Low	80	56 (70.0)	45.5 (20/44)	100 (36/36)	<0.01
LA penicillin	Low	81	30 (37.0)	33.3 (15/45)	41.7 (15/36)	0.49
Amoxicillin	Low	80	7 (8.8)	2.2 (1/44)	16.7 (6/36)	0.04
In-feed						
Virginiamycin	High	81	17 (21.0)	8.9 (4/45)	36.1 (13/36)	<0.01
Tylosin	Low	81	3 (3.7)	2.2 (1/45)	5.6 (2/36)	0.58
Oxytet/chlortetracycline	Low	80	12 (15.0)	2.2 (1/44)	30.6 (11/36)	<0.01
Monensin	No-human use	81	50 (61.7)	45.5 (20/45)	83.3 (30/36)	<0.01
Lasalocid	No-human use	81	5 (6.2)	4.4 (2/45)	8.3 (3/36)	0.65
Flavophospholipol	No-human use	78	10 (12.8)	0 (0/44)	29.4 (10/34)	<0.01

[a]There was no reported use of injectable neomycin, gentamicin and enrofloxacin or in-feed tilmicosin, trimethoprim-sulphonamide, salinomycin and narasin. [b]ASTAG antimicrobial importance ratings. [c]Count of respondents. P-values assess the significance of difference between the antimicrobial given and feedlot capacity. Small (<3000 cattle); large (>3000 cattle). ASTAG, Australian Strategic and Technical Advisory Group on Antimicrobial Resistance; LA, long-acting; SA, short-acting.

stewardship in this sector. For a group of beef feedlots of varying sizes geographically located across Australia (n = 83), we can report for the first-time comprehensive herd-level data for each antimicrobial used, including the administration routes, reasons for use and disease syndromes treated. Information on antimicrobial stewardship practices, including interactions with veterinarians, the use of protocols for veterinary treatments and inductions and the management of treated animals, are also reported.

Feedlot operators who responded to the survey relied predominantly on antimicrobials considered to be of low importance to human health according to the ASTAG ratings. The most commonly used antimicrobials were from the tetracycline, penicillin, macrolide and ionophore classes. Ceftiofur, a third-generation cephalosporin registered for use in beef cattle and rated by ASTAG as highly important,[6] was used by approximately one-third of feedlots. Most feedlots administered any injectable antimicrobial to fewer than 10% of animals, and for in-feed antimicrobials, to fewer than 20% of lots (pens) in the 12 months. Overall, the type and frequency of antimicrobials used in large feedlots were higher than small feedlots. Large feedlots also reported an increased frequency in veterinarian visits and more had veterinary treatment protocols compared with small feedlots. These results were expected given larger feedlots face greater health challenges associated with the co-mingling of large numbers of cattle from a wide variety of source herds, along with transportation of animals over long distances.[20] Overall, the preference for low importance antimicrobials and evidence that practices related to antimicrobial stewardship are underway is very encouraging.

Very few studies have been published internationally on antimicrobial use in beef feedlots.[12,21,22] Of those studies that have been published, the US NAHMS feedlot surveys from 1999,[16] 2011,[17] and 2017[18] are the most comprehensive and give an overview of antimicrobial use on US feedlots. For instance, according to the 2017 NAHMS survey,[18] 70.8% of US feedlots gave any (one or more) in-feed antimicrobial agent, with ionophores and chlortetracycline most commonly used. In the same period, it was reported that 80% of US feedlots treated individual animals with injectable antimicrobials, while 14.8% of feedlots treated cattle as a group with any injectable drug. For the first time in 2017, the NAHMS survey also reported on antimicrobial stewardship practices, with 79.7% of feedlots reporting the use of veterinary services and 80.5% reporting a veterinarian had visited the facility more than two times in the 12 months. However,

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Table 5. Proportion of feedlot respondents that treated cattle with any injectable antimicrobial, by antimicrobial and treatment purpose (individual animal treatment, mass animal treatment or timed treatment) in the previous 12 months

Antimicrobial[a]	ASTAG importance ranking[b]	n[c]	Users (%)	Frequency (%) of response		
				Individual treatment	Mass treatment[d]	Timed treatment[e]
SA ceftiofur	High	82	28 (34.1)	27 (96.4)	0 (0)	1 (3.6)
LA ceftiofur	High	82	28 (34.1)	26 (92.9)	0 (0)	2 (7.1)
Trimethoprim-sulphonamide	Medium	82	21 (25.6)	21 (100)	0 (0)	0 (0)
Tilmicosin	Low	81	27 (33.3)	15 (55.6)	11 (40.7)	1 (3.7)
Tulathromycin	Low	82	46 (56.1)	44 (95.6)	1 (2.1)	1 (2.1)
SA oxytetracycline	Low	82	47 (57.3)	45 (95.7)	1 (2.1)	1 (2.1)
LA oxytetracycline	Low	82	16 (19.5)	16 (100)	0 (0)	0 (0)
SA penicillin	Low	81	53 (65.4)	51 (96.2)	0 (0)	2 (3.8)
LA penicillin	Low	82	30 (36.5)	29 (96.7)	0 (0)	1 (3.3)
Amoxicillin	Low	81	7 (8.6)	7 (100)	0 (0)	0 (0)
Total users		83	68 (81.9)	—	—	—

[a]There was no reported use of injectable neomycin, gentamicin and enrofloxacin. [b]ASTAG antimicrobial importance ratings for humans. [c]Count of respondents. [d]Mass treatment in response to a disease outbreak (metaphylaxis). [e]Timed/scheduled treatment of lots (prophylaxis). More than one treatment purpose for an antimicrobial may have been indicated. ASTAG, Australian Strategic and Technical Advisory Group on Antimicrobial Resistance; LA, long-acting; SA, short-acting.

Table 6. Proportion of feedlot respondents that treated cattle with any in-feed antimicrobial, by antimicrobial and treatment purpose (mass animal treatment, timed treatment, preventative treatment or growth promotion) in the previous 12 months

In-feed antimicrobial[a]	ASTAG importance ranking[b]	n[c]	Users (%)	Frequency (%)			
				Mass treatment[d]	Timed treatment[e]	Preventative treatment[f]	Growth promotion[g]
Virginiamycin	High	82	17 (20.7)	0 (0)	0 (0)	12 (70.6)	5 (29.4)
Tylosin	Low	82	3 (3.7)	1 (33.3)	0 (0)	2 (66.7)	0 (0)
Oxytet/ chlortetracycline	Low	81	15 (18.5)	5 (33.3)	7 (46.7)	3 (20.0)	0 (0)
Monensin	Unrated	82	60 (73.1)	0 (0)	3 (5.0)	34 (56.7)	23 (38.3)
Lasalocid	Unrated	82	3 (3.7)	0 (0)	0 (0)	2 (66.7)	1 (33.3)
Flavophospholipol	Unrated	79	11 (13.9)	0 (0)	0 (0)	5 (45.5)	6 (54.5)
Total users		83	53 (63.9)				

[a]There was no reported use of in-feed tilmicosin, trimethoprim-sulphonamide, salinomycin or narasin. [b]AASTAG antimicrobial importance ratings. [c]Count of respondents. [d]Mass treatment in response to a disease outbreak (metaphylaxis). [e]Timed/scheduled treatment of lots (prophylaxis). [f]Preventative treatment long-term treatment to prevent disease. [g]Growth promotion, use of an antimicrobial to improve physiological performance. More than one treatment purpose may have been indicated. ASTAG, Australian Strategic and Technical Advisory Group on Antimicrobial Resistance; LA, long-acting; SA, short-acting.

caution should be exercised when comparing antimicrobial use between studies since study design, questionnaire administration, sample sizes and response rates are different. Also, factors such as drug registration status, non-veterinary access to antimicrobials, the prevalence of disease and management practices on feedlots are likely to be different between countries.

The respiratory syndrome, Bovine respiratory disease, is the primary cause of morbidity and death in feedlot cattle.[15,23] Respiratory infection, coupled with a range of complex animal and management factors, can lead to high morbidity and mortality.[24] In this survey, respiratory disease was the most commonly reported syndrome requiring treatment with antimicrobials. Most respondents indicated the use of antimicrobials such as injectable tulathromycin, injectable SA oxytetracycline and in-feed oxytetracycline/ chlortetracycline, which are considered to be of low importance to human health according to the ASTAG ratings. Feedlots also reported the use of SA and LA formulations of ceftiofur for the treatment of cattle with respiratory disease, a registered use for this compound. However, three feedlots indicated the use of ceftiofur for timed (scheduled) treatments, and several respondents indicated ceftiofur was used to

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Table 7. Proportion of feedlot respondents that treated cattle with any injectable or in-feed antimicrobial and the disease syndromes treated in the previous 12 months

Antimicrobial[a]	ASTAG importance ranking[b]	n[c]		Respiratory	Gastrointestinal	Musculoskeletal	Neurological	Urogenital	Other
Injectable									
SA ceftiofur	High	28	% within column	40.0%	4.8%	10.7%	4.8%	5.7%	0%
			% within row	85.7%	3.6%	21.4%	3.6%	7.1%	0%
			Count	24	1	6	1	2	0
LA ceftiofur	High	28	% within column	45.0%	9.5%	5.4%	14.3%	2.9%	22.2%
			% within row	96.4%	7.1%	10.7%	10.7%	3.6%	7.1%
			Count	27	2	3	3	1	2
Trimethoprim-sulphonamides	Medium	21	% within column	0%	85.7%	0%	9.5%	5.7%	0%
			% within row	0%	85.7%	0%	9.5%	9.5%	0%
			Count	0	18	0	2	2	0
Tilmicosin	Low	27	% within column	41.7%	0%	1.8%	4.8%	0%	0%
			% within row	92.6%	0%	3.7%	3.7%	0%	0%
			Count	25	0	1	1	0	0
Erythromycin	Low	2	% within column	1.7%	0%	1.8%	0%	0%	0%
			% within row	50.0%	0%	50.0%	0%	0%	0%
			Count	1	0	1	0	0	0
Tulathromycin	Low	46	% within column	71.7%	9.5%	3.6%	4.8%	2.9%	11.1%
			% within row	93.5%	4.3%	4.3%	2.2%	2.2%	2.2%
			Count	43	2	2	1	1	1
SA oxytetracycline	Low	47	% within column	63.3%	28.6%	37.5%	66.7%	17.1%	22.2%
			% within row	80.9%	12.8%	44.7%	29.8%	12.8%	4.3%
			Count	38	6	21	14	6	2
LA oxytetracycline	Low	16	% within column	18.3%	14.3%	21.4%	23.8%	17.1%	11.1%
			% within row	68.8%	18.8%	75.0%	31.3%	37.5%	6.3%
			Count	11	3	12	5	6	1
Florfenicol	Low	2	% within column	3.3%	0%	0%	4.8%	0%	0%
			% within row	66.7%	0%	0%	33.3%	0%	0%
			Count	2	0	0	1	0	0
SA penicillin	Low	53	% within column	5.0%	38.1%	82.1%	38.1%	74.3%	77.8%
			% within row	5.7%	15.1%	86.8%	15.1%	49.1%	13.2%
			Count	3	8	46	8	26	7
LA penicillin	Low	31	% within column	3.3%	0%	48.2%	4.8%	28.6%	0%
			% within row	6.5%	0%	87.1%	3.2%	32.2%	0%
			Count	2	0	27	1	10	0
Amoxicillin	Low	7	% within column	8.3%	4.8%	5.4%	4.8%	5.7%	0%
			% within row	71.4%	14.3%	42.9%	14.3%	28.6%	0%
			Count	5	1	3	1	2	0
Total injectable use: Feedlots		68	% within row	88.2%	23.7%	63.6%	23.7%	51.5%	13.2%
			Count	60	21	56	21	35	9
In-feed antimicrobials									
Virginiamycin	High	16	% within column	15.8%	32.1%	28.6%	50.0%	33.3%	28.6%
			% within row	18.7%	56.3%	12.5%	12.5%	6.3%	11.8%
			Count	3	9	2	2	1	2
Tylosin	Low	3	% within column	10.5%	14.3%	14.3%	0%	0%	0%
			% within row	66.7%	33.3%	33.3%	0%	0%	0%
			Count	2	1	1	0	0	0

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Table 7. Continued

Antimicrobial[a]	ASTAG importance ranking[b]	n[c]		Respiratory	Gastrointestinal	Musculoskeletal	Neurological	Urogenital	Other
Oxytet/ chlortetracycline	Low	12	% within column	57.9%	0%	28.6%	0%	0%	0%
			% within row	91.7%	0%	16.7%	0%	0%	0%
			Count	11	0	2	0	0	0
Monensin	Unrated	50	% within column	21.1%	96.4%	42.9%	75.0%	100%	85.7%
			% within row	8.0%	54.0%	6.0%	6.0%	6.0%	12.0%
			Count	4	27	3	3	3	6
Lasalocid	Unrated	5	% within column	0%	7.1%	0%	0%	0%	0%
			% within row	0%	40.0%	0%	0%	0%	0%
			Count	0	2	0	0	0	0
Flavophospholipol	Unrated	10	% within column	0%	14.3%	0%	0%	0%	14.3%
			% within row	0%	40.0%	0%	0%	0%	10.0%
			Count	0	4	0	0	0	1
Total in-feed use: Feedlots		53	% within row	35.8%	52.8%	13.2%	7.5%	5.7%	13.2%
			Count	19	28	7	4	3	7

[a]There was no reported use of injectable tylosin, neomycin, gentamicin and enrofloxacin or in-feed tilmicosin, trimethoprim-sulphonamide, salinomycin and narasin. [b]ASTAG antimicrobial importance ratings. [c]Count of respondents. More than one disease syndrome may have been treated with any antimicrobial. ASTAG, Australian Strategic and Technical Advisory Group on Antimicrobial Resistance; LA, long-acting; SA, short-acting.

treat diseases of the gastrointestinal, musculoskeletal, neurological and urogenital systems. In Australia, ceftiofur is registered in cattle for individual animal treatment only, and there are restrictions on its use for conditions other than respiratory diseases and in other animal species. In the USA, ceftiofur does not have the same requirements for individual use only, and the compound is registered to treat a range of diseases, including respiratory disease, foot rot and metritis. In 2017, 2.7% of all US feedlots used ceftiofur for group treatment (>90% animals in a pen), and 8.7% of large feedlots (≥1000 cattle) used the compound for group treatment.[18]

Most antimicrobials are prescription-only schedule 4 medicines in Australia. Consequently, only a veterinarian can prescribe schedule 4 antimicrobials, or a pharmacist, acting under instruction from a veterinarian, may dispense schedule 4 antimicrobials to an animal owner. Legislation in each State and Territory imposes varying obligations on veterinarians to have a *bonafide* client relationship before supplying prescription drugs, with a minimum requirement of a working knowledge of the farming enterprise to which the drugs are supplied. Legislation describes the conditions for off-label use of registered veterinary medicines and the use of unregistered chemicals in food animals. In most States and Territories, off-label use of registered veterinary medicines is permitted in a single food animal under written direction from a veterinarian. In this study, some respondents indicated the use of ceftiofur and virginiamycin for disease conditions other than those stated on the product label. However, the questionnaire did not specifically ask respondents if the use of an antimicrobial in a way other than stated on the label was done under the direction of a veterinarian. Therefore, these responses cannot be further interpreted to determine if antimicrobial use patterns are compliant with legislative requirements. Further investigation and clarification of this reported usage pattern must be followed-up through the adoption of industry-wide antimicrobial stewardship guidelines. Clustering, concerning the antimicrobial usage reported by respondents, was considered to have limited effect in this study. In Australia, the strict regulatory framework that controls chemical use in cattle, the restricted number of registered antimicrobials, and obligations related to withholding periods, export slaughter intervals and importing country requirements before cattle can be slaughtered limit the antimicrobial options available for use in feedlot cattle irrespective of geographic location, population strata and veterinarian preferences.

The scarcity of data on antimicrobial use and practices in animals in many countries is a well-recognised limitation in the management of antimicrobial resistance. However, the most appropriate method to collect these data has not been determined. In this study, we utilised a mailed questionnaire, and in doing so, identified important shortfalls when using this method to collect data on complex and sensitive issues. For valid inferences to be drawn about the population of interest from the information collected by questionnaire, respondents must be representative of the population from which they are derived, and answers given must be accurate.[25,26] However, in this study, a major limitation was the low response rate. While a low response rate does not necessarily negate the contribution of the findings to antimicrobial stewardship it does impose the need to allow for a higher likelihood of non-response bias.[25] Non-response bias is a well-known concern when using mailed questionnaires for data collection. Mailed questionnaires are also vulnerable to selection bias since participation is self-

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determined, with people less engaged in the topic less likely to respond. In this study, respondents were self-selected, so selection bias has likely influenced the findings. For example, respondents who are aware of antimicrobial resistance may have been more likely to engage in discussions regarding the judicious use of antimicrobial agents than those who did not respond. When non-response and selection biases exist and cannot be controlled, inferences about the broader population are not as reliable.

To collect data on antimicrobial use and stewardship practices on feedlots, the questionnaire used in this study was lengthy, complex and sought information that may be perceived as sensitive. Previous studies have demonstrated that respondents are more likely to disclose sensitive information in anonymous mailed questionnaires than in other survey methods.[25,27,28] However, the length and complexity of a questionnaire are also known to substantially influence the response rate by creating a cognitive burden on potential respondents. The complexity of the questionnaire use in this study may have resulted in response and prevarication bias, where respondents may not have accurately answered questions. Response and prevarication bias were managed by guaranteeing the anonymity of respondents' identity, although this does not entirely eliminate the possibility of such bias given the controversy surrounding antimicrobial use in food animals. Recall bias is also a common problem with self-administered questionnaires and may have occurred given the reliance on the accuracy of chemical records kept by feedlots over 12 months. There are obvious limitations in undertaking post-response follow-up to clarify unexpected or contradictory responses given the anonymous nature of the questionnaire. There is also a possibility that responses may have been influenced by the information pack accompanying the questionnaire, which contained general information on antimicrobial use in the cattle industry. Also, the questionnaire did not differentiate respondents based on membership of the National Feedlot Accreditation Scheme, the industry quality assurance scheme. A key component of the scheme is related to chemical use and record keeping. Hence, respondents who participate in the National Feedlot Accreditation Scheme are more likely to be familiar with responsible chemical use than those who do not participate in the scheme. To facilitate interpretation of results, future studies should determine the proportion of respondents who are members of quality assurance programs.

Survey results are unadjusted for feedlot population differences in each State and Territory as there are no recent feedlot statistics available. The available data report the number of cattle on grain rather than the number of feedlots by state or territory. However, a range of feedlot size capacities was represented in the survey responses, indicating a reasonable coverage of feedlot enterprises present in Australia. Further, the questionnaire did not ascertain if the feedlot operations of respondents met the specific criteria for a beef feedlot as defined by the National Feedlot Accreditation Scheme,[19] although much of the eligible population of respondents was derived from the clientele lists of beef feedlot consultant veterinarians who consult in multiple States and Territories.

More reliable methods of survey delivery should be developed for the ongoing collection of antimicrobial use data at the feedlot-level. Depending on the information sought, other survey methods including face-to-face interviews with interrogation of treatment records as is performed in Canada and the USA,[18,29] Web-based surveys, or smartphone apps may increase the response rate and quality of the data available for analysis. Alternatively, the use of software that can extract continuous near real-time data from computerised farm records or veterinary clinic records could provide the data required for the ongoing surveillance of antimicrobial use in feedlots. However, before undertaking further surveys at the feedlot-level, consideration should be given to the type of data required to inform prescribing guidelines and importantly, quantify antimicrobial use for national surveillance.

One of the key benefits of the questionnaire method is its value in informing behaviours and driving social change. And so, while inferences to the broader population of beef feedlots in Australia are not possible, the data presented here has been valuable in supporting the beef feedlot industry's development of antimicrobial stewardship guidelines[30] and has acted as a starting point for other Australian livestock sectors to evaluate antimicrobial use.

Conclusion

This is the first comprehensive survey of antimicrobial use in Australian beef feedlots. The operation-level descriptive estimates of antimicrobial usage described here are a starting point for further research aimed at generating accurate quantitative estimates of antimicrobial use at the animal level, and for identifying veterinary and owner motivations for antimicrobial use. Ideally, surveillance of antimicrobial usage would occur alongside surveillance of antimicrobial resistance, thereby enabling the design and implementation of optimal strategies to control antimicrobial resistance in the beef feedlot sector.

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References

1. World Health Organisation. *WHO global strategy for containment of antimicrobial resistance*. Geneva, WHO, 2001.
2. OIE. *Resolution 26. Combating antimicrobial resistance and promoting the prudent use of antimicrobial agents in animals*. 83rd General Assembly, Paris, 2015. Available at: http://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/AMR/A_RESO_AMR_2015.pdf. Accessed 22 November 2016.

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3. United Nations. *At UN, global leaders commit to act on antimicrobial resistance. Collective effort to address a challenge to health, food security and development*. Joint News Release, 21 September 2016. OPGA/WHO/FAO/OIE, New York, 2016. Available at: http://www.who.int/mediacentre/news/releases/2016/commitment-antimicrobial-resistance/en/. Accessed 22 September 2016.
4. OIE. *OIE annual report on the use of antimicrobial agents intended for use in animals: better understanding of the global situation*, 3rd report. OIE, Paris, 2018.
5. Australian Pesticides and Veterinary Medicines Authority. *Quantity of antimicrobial products sold for veterinary use in Australia: July 2005 to June 2010, 2014*. Available at: http://www.apvma.gov.au/. Accessed 08 September 2018.
6. Australian Strategic and Advisory Group on Antimicrobial Resistance. *Importance ratings and summary of antibacterial uses in human and animal health in Australia, version 1.0 commonwealth of Australia*, Canberra, 2018.
7. Australian Pesticides and Veterinary Medicines Authority. *Antibiotic resistance in animals: a report for the APVMA*, 2017. Available at: https://apvma.gov.au/sites/default/files/publication/27326-final_amr_report-_for_publishing_v02_140817_a939399.pdf. Accessed 26 August 2018.
8. World Organisation for Animal Health. *Chapter 6.10. Responsible and prudent use of antimicrobial agents in veterinary medicine. Terrestrial Animal Health Code*. Paris, OIE, 2018.
9. Australian Veterinary Association. *Guidelines for prescribing, authorising and dispensing veterinary medicines*, 2005. Available at: http://www.ava.com.au/sites/default/files/documents/Other/Guidelines_for_prescribing_authorising_and_dispensing_veterinary_medicines.pdf. Accessed 27 September 2017.
10. Meat and Livestock Australia. *Fast facts: Australian beef industry*, 2018. Available at: https://www.mla.com.au/globalassets/mla-corporate/prices–markets/documents/trends–analysis/fast-facts–maps/mla_beef-fast-facts-2018.pdf. Accessed 16 June 2019.
11. Meat and Livestock Australia. *Lot feeding brief: results for the June quarter 2018 feedlot survey*, 2018. Available at: https://www.mla.com.au/globalassets/mla-corporate/prices–markets/documents/trends–analysis/lot-feeding-brief/mla_lot-feeding-brief_august-2018.pdf. Accessed 26 August 2018.
12. Cameron A, McAllister TA Antimicrobial usage and resistance in beef production. *J Anim Sci Biotechnol* 2016;7:68.
13. Jan JS, McIntosh WA, Dean W et al. Predictors of differences in the perception of antimicrobial resistance risk in the treatment of sick, at-risk, and high-risk feedlot cattle. *Prev Vet Med* 2012;106:24–33.
14. Prescott JF History and current use of antimicrobial drugs in veterinary medicine. *Microbiol Spectr* 2017;5(6). https://doi.org/10.1128/microbiolspec.ARBA-0002-2017.
15. Hay KE, Morton JM, Schibrowski ML et al. Associations between prior management of cattle and risk of bovine respiratory disease in feedlot cattle. *Prev Vet Med* 2016;127:37–43.
16. United States Department of Agriculture. In: APHIS, editor. *National Animal Health Monitoring System (NAHMS) Feedlot 1999, Part III: health management and biosecurity in U.S. feedlots*. USDA, Fort Collins, 2000.
17. United States Department of Agriculture. *Feedlot 2011 part 1: management practices on U.S. feedlots with a capacity of 1,000 or more head*. Fort Collins, USDA-APHIS-VS-CEAH-NAHMS, 2013.
18. United States Department of Agriculture. *Antimicrobial use and stewardship on U.S. feedlots, 2017*. USDA-APHIS-VS-CEAH-NAHMS, Fort Collins, CO, 2019.
19. AUS-MEAT. *National feedlot accreditation scheme, rules and standards of accreditation handbook*, 2017. https://www.ausmeat.com.au/WebDocuments/NFAS_-_Rules_and_Standards_of_Accreditation.pdf. Accessed 9 April 2018.
20. Hay KE, Barnes TS, Morton JM et al. Risk factors for bovine respiratory disease in Australian feedlot cattle: use of a causal diagram-informed approach to estimate effects of animal mixing and movements before feedlot entry. *Prev Vet Med* 2014;117:160–169.
21. Green AL, Carpenter LR, Edmisson DE et al. Producer attitudes and practices related to antimicrobial use in beef cattle in Tennessee. *J Am Vet Med Assoc* 2010;237:1292–1298.
22. Carson CA, Reid-Smith R, Irwin RJ et al. Antimicrobial use on 24 beef farms in Ontario. *Can J Vet Res* 2008;72:109–118.
23. Hilton WM BRD in 2014: where have we been, where are we now, and where do we want to go? *Anim Health Res Rev* 2014;15:120–122.
24. Snowder GD, Van Vleck LD, Cundiff LV et al. Bovine respiratory disease in feedlot cattle: environmental, genetic, and economic factors. *J Anim Sci* 2006;84:1999–2008.
25. Murdoch M, Simon AB, Polusny MA et al. Impact of different privacy conditions and incentives on survey response rate, participant representativeness, and disclosure of sensitive information: a randomized controlled trial. *BMC Med Res Methodol* 2014;14:90.
26. Dohoo I, Martin W, Stryhn H. *Veterinary epidemiologic research*. 2nd edition. Charlottetown, VER Inc, 2009.
27. Rocheleau CM, Romitti PA, Sherlock SH et al. Effect of survey instrument on participation in a follow-up study: a randomization study of a mailed questionnaire versus a computer-assisted telephone interview. *BMC Public Health* 2012;12:579.
28. Bowling A Mode of questionnaire administration can have serious effects on data quality. *J Public Health (Oxf)* 2005;27:281–291.
29. Government of Canada. *Canadian integrated program for antimicrobial resistance surveillance (CIPARS) 2015 annual report*. Guelph, Public Health Agency of Canada, 2017.
30. Meat and Livestock Australia. *Antimicrobial stewardship guidelines for the Australian cattle feedlot industry*, 2018. Available at: https://www.mla.com.au/globalassets/mla-corporate/research-and-development/program-areas/animal-health-welfare-and-biosecurity/mla_antimicrobial-stewardship-guidelines.pdf. Accessed 22 May 2019.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site: http://onlinelibrary.wiley.com/doi/10.1111/avj.12889/suppinfo.

Data S1. Supplementary Material: Questionnaire: Types of antimicrobial agents used and reasons for use in beef cattle kept at feedlots in the previous 12-month period in Australia.

Table S1. Proportion of feedlot respondents that treated cattle with any injectable or in-feed antimicrobials, by antimicrobial given and by feedlot capacity (small, medium, large) in the previous 12 months. P-values assess the significance of difference between the antimicrobial given and feedlot capacity. LA, long-acting; SA, short-acting.

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Supplementary materials for Chapter 5 can be found in Appendix 4

Chapter 6:

General Discussion

General Discussion

This thesis examined the quality of measurement systems used to evaluate antimicrobial resistance and antimicrobial use in animal populations. The four studies in this thesis expand our understanding of the data generated from phenotypic antimicrobial susceptibility tests (Chapters 2, 3, 4) and from questionnaires used to obtain information on antimicrobial use (Chapter 5).

The quality of information derived from surveillance activities is strongly dependent on accuracy and reliability – of the sampling methodology and the measurement tools used to collect information. However, for animals, the objectives of national programs are often poorly defined, the sampling and testing protocols may not be purposefully designed, and surveillance outputs can fall short of providing the information required to guide policies. As exponentially more research and surveillance activities are undertaken on antimicrobial resistance, there is a strong need for critical appraisal of the quality, usefulness, and credibility of the data generated. For instance, what are the objectives of the research or surveillance activity? What decisions do these objectives support? And, will the data properly inform decision-making? Research and other information-gathering activities cannot be considered surveillance if their only goal is to collect data for its intrinsic value. The aim of surveillance should be the ongoing collection of data to guide policy and actions, and in doing so, needs standardised and accepted methods and a strong understanding of the performance of the tests used to generate the data.

This discussion presents an opportunity to examine the findings of each research area more thoroughly so their inferences can be addressed, and future directions proposed.

Is disc diffusion accurate for use in national surveillance programs?

Phenotypic assays are the backbone of national programs for surveillance of antimicrobial resistance. This is especially so in resource-limited settings such as the veterinary sector, where there has been slow adoption of genetic and molecular technologies. At present, the performance of phenotypic assays is poorly defined in both human and animal settings. This raises questions about the quality of information reported by national surveillance programs. Thus, the accuracy and reliability of phenotypic susceptibility data must be evaluated, and this thesis does this.

Chapters 2 and 3 evaluated the diagnostic test accuracy of disc diffusion relative to broth microdilution (the accepted reference standard) for a range of antimicrobial classes and two important bacterial pathogens of animals, *E. coli* (Chapter 2) and *S. pseudintermedius* (Chapter 3). Where Chapter 2 evaluated the diagnostic accuracy of disc diffusion relative to broth microdilution, Chapter 3 built on this approach by assessing both disc diffusion and broth microdilution to real-time PCR to determine methicillin resistance. Few studies have evaluated broth microdilution to assays that can be assumed to be more accurate. However, as genetic and molecular technologies become more widely available, opportunities exist to evaluate broth microdilution. Indeed, the evidence presented in Chapter 3 demonstrates this process for *S. pseudintermedius* (a ubiquitous opportunistic pathogen of dogs).

For most antimicrobials evaluated, disc diffusion was found to be accurate at predicting the antimicrobial susceptibility of clinical *E. coli* and *S. pseudintermedius* that could otherwise be determined by broth microdilution. The ability of disc diffusion to correctly classify isolates, relative to broth microdilution, varied with the antimicrobial and clinical breakpoint used to dichotomise the data. For example, disc diffusion performed strongly for critically important antimicrobial classes such as fluoroquinolones (e.g., ciprofloxacin) and third generation cephalosporins (e.g., ceftiofur) and first-line antimicrobials such as tetracycline and ampicillin. Therefore, the acquisition of data generated by disc diffusion testing in veterinary laboratories

could be beneficial in informing our understanding of antimicrobial resistance in animal health and public health. However, overlapping populations of susceptible and resistant isolates resulted in inferior estimates for some antimicrobials, made worse by the clinical breakpoint used to determine status. For example, disc diffusion performed less favourably for amoxicillin, amoxicillin-clavulanic acid, and trimethoprim-sulfamethoxazole, where susceptible and resistant populations were shown to overlap.

The reliance on using a threshold value to dichotomise continuous data presents a strong justification for using ROC analysis to evaluate test accuracy, although very few diagnostic test evaluation studies have done so. ROC has limitations when the reference test is less than perfect, as is the case with broth microdilution. More robust estimates of test performance can be obtained by using a more accurate reference test (as occurred in Chapter 3) or preferably, by using latent class analysis which is not reliant on a perfect reference test (Enoe, Georgiadis & Johnson 2000; Johnson, Jones & Gardner 2019; Pepe & Janes 2007). However, the use of a more accurate reference test is based on there being such a test in existence and that it is affordable and accessible, while for the studies reported in this thesis, assumptions which underlie latent class analysis could not be met.

Notwithstanding these limitations, the use of an extensive national collection of clinical *E. coli* (n=994) and *S. pseudintermedius* (n=614) isolates has greatly strengthened the analysis of phenotypic assays. Indeed, few national collections from veterinary sources have been reported elsewhere and which are comparable or superior in size and geographic representativeness. Therefore, the performance estimates reported in the thesis can be considered sufficiently robust such that the relative diagnostic sensitivity and specificity of disc diffusion for a range of antimicrobials to *E. coli* and *S. pseudintermedius* can be co-opted for use in surveillance to correct for true prevalence, thereby allowing comparison with prevalence estimates generated from broth microdilution data.

The ability of phenotypic assays to yield discrepant results was highlighted in Chapters 2 and 3 and became a focus for further investigation in Chapter 4. These disagreements are preserved in the analyses to faithfully reflect the measurement error inherent in all tests and conditions that arise in diagnostic laboratories. However, very often in microbiological research, the solution to resolving a disagreement is to retest isolates to confirm 'true' status using a reference test (e.g., broth microdilution) and or a 'resolver' test (e.g., PCR). This approach, known as discrepant analysis, usually results in an overestimation of the performance of a comparator test (Green, Black & Johnson 1998; Miller 2012). That is, where both test results agree, the status of an isolate is considered 'true'; however, where two test results disagree, re-testing shifts a measurement from disagreement to agreement. Resolving disagreement between measurements by repeat testing should be discontinued. If we are to truly understand the validity of these data, a more rigorous approach to the evaluation of diagnostic test performance is needed, similar to that undertaken in Chapters 2 and 3, or by latent class analysis when the underlying assumptions can be met (Johnson, Jones & Gardner 2019).

How precise is disc diffusion when used in veterinary diagnostic laboratories?

Anecdotally, it appears few technicians performing and interpreting phenotypic assays understand what level of variability they can expect if the same isolate is assessed on multiple occasions. Chapter 4, therefore examined the intra-laboratory agreement (repeatability) and inter-laboratory agreement (reproducibility) of disc diffusion in veterinary diagnostic laboratories to measure this variability and to understand whether the assay has a role in the surveillance of antimicrobial resistance. Repeatability and reproducibility estimates provide a practical interpretation of the extent of variation in zone diameter measurements expected in diagnostic laboratories when testing the same isolate.

The precision of disc diffusion was found to be satisfactory, although the extent of variation recorded for some antimicrobials, including ampicillin and trimethoprim-sulfamethoxazole, was of concern. Measurement variation is critical the closer the zone diameter measurement is to the clinical breakpoint, where the likelihood of misclassification is high. This was seen in Chapter 4 for isolates with marginal susceptibility (e.g., ampicillin) and for antimicrobials where zone edges were difficult to read (e.g., trimethoprim-sulfamethoxazole). Therefore, when defining which antimicrobials to include in testing panels for surveillance, a critical consideration should be the ability of those antimicrobials to yield reliable zone diameters, especially when there are inherent difficulties in reading zone edges. While this thesis did not examine sources contributing to the variation seen in zone diameters, it is postulated that errors in manual measurement make a substantial contribution to this variation (Hombach, Zbinden & Bottger 2013; Idelevich et al. 2016). Consequently, the adoption of automated zone readers to reduce variation associated with visual reading is a recommendation of this thesis.

Interpretation of diagnostic test performance for use in surveillance

While the findings reported in Chapters 2, 3, and 4 are a product of interactions that occurred under the research conditions described in this thesis, they can be generalised to the broader population of veterinary laboratories, provided consideration is given to performance estimates of the assay. For instance, disc diffusion performs better for some antimicrobials than others. This knowledge can be used to design antimicrobial panels for the surveillance of bacterial pathogens in animals that reflect antimicrobials that can be (i) reliably measured, and (ii) are of interest to animal health. Arising out of the research findings from this thesis is a list of recommended antimicrobials for inclusion in disc diffusion testing of *E. coli* and *S. pseudintermedius* clinical isolates from animals (Table 1).

Antimicrobials that performed strongly across CLSI susceptible and resistant clinical breakpoints are recommended for inclusion, including ampicillin, ceftiofur, ciprofloxacin, and tetracycline. Note, ciprofloxacin was used in this thesis as a representative of the fluoroquinolone class as it is commonly used in national surveillance owning to its relevance to public health. However, there is a need to evaluate the performance of other fluoroquinolone class members specific to animal health, including enrofloxacin and marbofloxacin which are important for therapeutic decision-making. Antimicrobials with variable performance require further consideration of performance estimates before inclusion. For example, for cefovecin and gentamicin, disc diffusion performance was much stronger when the resistant breakpoint was applied compared to the susceptible breakpoint. Antimicrobials that performed poorly across both breakpoints, namely amoxicillin-clavulanic acid and cefoxitin, are not recommended for inclusion in antimicrobial panels. The recommendation to exclude amoxicillin-clavulanic acid from disc diffusion testing on *E. coli* and *S. pseudintermedius* clinical isolates may have ramifications for antimicrobial susceptibility testing in veterinary laboratories, given the frequent use of this drug in small animal medicine.

Table 1. Recommended composition of disc diffusion antimicrobial panels for *Escherichia coli* and *Staphylococcus pseudintermedius* clinical isolates from animals.

Inclusion in antimicrobial panel	***Escherichia coli***	***Staphylococcus pseudintermedius***
Yes	ampicillin, ceftiofur, ciprofloxacin, tetracycline	chloramphenicol, ciprofloxacin[†], clindamycin, oxacillin, tetracycline
No	amoxicillin-clavulanic acid, cefoxitin	amoxicillin-clavulanic acid, cefoxitin, cephalothin
Requires further consideration	cefovecin, cephalothin, gentamicin, trimethoprim-sulfamethoxazole	cefovecin, rifampicin
Unable to determine*	amikacin, imipenem	

[†] Ciprofloxacin is representative of the fluoroquinolone class, however, there is a need to evaluate the performance of other fluoroquinolone class members specific to animal health, including enrofloxacin and marbofloxacin.

[*]All isolates were susceptible to the antimicrobial.

Knowledge of the extent of laboratory-to-laboratory variation in the measurement of zone diameters is not only valuable for participation in surveillance programs, but this information can also be used to standardise the disc diffusion assay further to minimise measurement error. Indeed, the findings reported in this thesis support the hypothesis that susceptibility results from disc diffusion data, as it is generated in veterinary laboratories, can contribute to national surveillance programs for animals. However, before this major finding can be acted upon, the logistics of acquiring data from diagnostic laboratories need to be further considered. Specifically, assessment of the quality of susceptibility data and the laboratory information management systems (LIMS) used in diagnostic laboratories must be undertaken since the data required for a surveillance system is different from what is necessary for clinical diagnosis. Further, issues associated with the highly selective nature of clinical submissions, potentially missing data on important epidemiological covariates (e.g., demographic data), and standardisation of test protocols also need to be studied. Diagnostic stewardship, championed by WHO (2017a), with its key objective of providing accurate and representative data on antimicrobial resistance, and the establishment of 'surveillance sites' responsible for collecting data at the local level, are important concepts which should be actively pursued in animal health.

How well do questionnaires perform to collect farm-level antimicrobial usage data?

The management of antimicrobial resistance is aided by the surveillance of two characteristics of the population of bacteria and animals. Firstly, the collection of diagnostic data on resistance in bacterial pathogens or commensal organisms, and secondly, the collection of data on the use of antimicrobial agents via questionnaires or other survey tools. There are many potential sampling points for the collection of information on antimicrobial use but of interest to this thesis is data collected at the farm-level. Farm-level data provides information on actual antimicrobial use as opposed to veterinary-clinic level data, which only provides information on the prescription of antimicrobial agents. As such, farm-level data is invaluable in the formulation of relevant, industry-specific antimicrobial stewardship programs.

In Chapter 5, the beef feedlot sector was used as a case study to examine the usefulness of a common survey method (i.e., self-administered mailed questionnaire) to obtain information on antimicrobial use at the farm-level. This survey process, when used to collect data on livestock sectors, is expected to be driven by industry and, consequently, faces several methodological constraints including different stakeholder needs, budget restrictions, and variable farmer engagement.

The questionnaire administered in Chapter 5 included a complex array of questions on the antimicrobial classes used on-farm in the previous twelve months, purposes of use, disease syndromes, and proportions of animals treated. Overall, the use of antimicrobial agents reported by respondents was determined to be appropriate for the purpose indicated, and there was a strong preference for antimicrobial classes of low importance, or not used, in human medicine including tetracyclines, penicillins, macrolides, and ionophores. However, a major limitation of this study was the low response rate (16%). While a low response rate does not necessarily equate to data that cannot add useful understanding, the risk of non-response bias is higher (Murdoch et al. 2014). The complexity of a questionnaire substantially influences the response rate and may also impact on the quality of information collected. For example, a questionnaire that is lengthy, complex, and asks many commercially, legally, and socially sensitive questions are unlikely to garner a high response rate. This represents a lost opportunity to collect data needed to inform decision-making. Indeed, the questionnaire used in Chapter 5 is a case study in the development of a survey instrument that was not well optimised for its purpose. The final design of this questionnaire was the product of multiple stakeholder involvement, coupled with poorly defined objectives for conducting the survey. As a result, control over the design of the survey was lost in the desire to obtain as much information as possible. Further, the complexity of the questionnaire likely placed a considerable cognitive burden on potential respondents, which contributed to the low response rate.

When designing questionnaires intended for farmers, the mistakes made in this survey should be avoided. More reliable methods of survey delivery should be considered for the on-going collection of antimicrobial use data at the farm-level. Other survey methods may increase the response rate and usefulness of the data for analysis. Before undertaking surveys at the farm-level, further consideration should be given to the type and quantity of data required for analysis and the reporting of antimicrobial use to the OIE (OIE 2017). Future surveys should also look to quantify antimicrobial use to allow for the calculation of metrics which not only meet OIE reporting requirements but can also be used to inform prescribing guidelines.

A key benefit of questionnaires is its value in informing behaviours and driving social change. And so, while the response rate experienced in this study meant we were unable to make inferences to the wider population, the data has been valuable in supporting the beef feedlot industry’s development of antimicrobial stewardship guidelines and a training program (Meat & Livestock Australia 2018) and has also acted as a starting point for other livestock sectors to evaluate their antimicrobial use. This is an example of participatory research whereby industry leaders, farmers, researchers, and government can come together to enhance data collection and analysis in order to design tools that drive positive change.

The utility of the Antimicrobial agent Use Risk Matrix

Communicating complex issues such as antimicrobial stewardship is challenging given the range of factors influencing antimicrobial use in food animals. Simple communication tools that engage farmers and veterinarians are essential in driving change in behaviours. One such communication tool I developed as a result of Chapter 5 of this thesis is the Antimicrobial agent Use Risk Matrix for beef feedlots (Figure 1). The risk assessment matrix categorises the risk of antimicrobial use practices at individual feedlots based on an overall assessment of behaviours and antimicrobial-associated factors. Behaviours include whether a veterinary treatment protocol has been issued, correct dosing and treatment periods are observed, and records are maintained. Antimicrobial-associated factors include the proportion of animals treated in the past 12 months, the importance ranking of antimicrobial use, and route of administration. Scores are assigned to each element, and the overall score is plotted onto the matrix, identifying the category of risk associated with the feedlot. Weightings are assigned to an antimicrobial agent's importance rating according to ASATG and the route of administration. A detailed explanation of the weightings applied in the index can be found in Appendix 5.

There are many advantages to using a risk matrix: it can be made available in different formats, it presents complex data in a visual form, it is easily adaptable if risk factors change (e.g., importance rankings), and it can be used as a benchmarking tool. The Antimicrobial agent Use Risk Matrix could also be a very useful tool for designing risk-based surveillance activities in food animals, where strata of animals are sampled according to their exposure to antimicrobial agents and the behaviours of those who care for them. However, there are limitations to using risk matrices – the assignment of risk can be subjective and may assign high risk to factors which are a small risk or vice versa, and the index may oversimplify the complexity of antimicrobial use on farms. Notwithstanding these limitations, the purpose of the Antimicrobial agent Use Risk Matrix is to be an interactive tool which engages farmers and veterinarians on the complex issues associated with antimicrobial resistance and use.

		Antibiotic Use Behaviours				
		Vet treatment protocol, vet instructions followed, animals weighed prior to treatment, correct dose, route, administration, treatment recorded	Vet treatment protocol, vet instructions mostly followed, animals weighed prior to treatment, correct dose, route, administration, treatment recorded	Vet treatment protocol, vet instructions mostly followed, animals not weighed prior to treatment, always/ mostly correct dose, route, administration, always/ mostly treatment recorded	+/- vet treatment protocol, vet instructions may be followed, animals not weighed prior to treatment, correct dose, route, administration not followed, no treatment records	No vet treatment protocol, vet instructions not followed, animals not weighed prior to treatment, Unknown if correct dose, route, administration, no treatment records
Antibiotic Use		**Optimal**	**Adequate**	**Suboptimal**	**Inadequate**	**Non-compliant**
>20% animals treated in past 12 months, low, medium, high importance drugs used, injectable and in-feed administration	**High risk**	High	High	Extreme	Extreme	Extreme
10-20% animals treated in past 12 months, low, medium, high importance drugs used, injectable and in-feed administration	**Medium risk**	Medium	Medium	High	High	High
2-10% animals treated in past 12 months, low, medium, +/- high importance drugs used, injectable use, unrated in-feed administration	**Some risk**	Medium	Medium	Medium	Medium	High
<2% animals treated in past 12 months, low, medium, +/- high importance drugs used, injectable use, +/- unrated in-feed administration	**Low risk**	Low	Low	Medium	Medium	Medium
No antibiotics used in past 12 months	**Negligible risk**	Low	Low	Low	Low	Low

Figure 1. Antimicrobial agent Use Risk Index for beef feedlots based on the risk associated with the behaviour towards antimicrobial agent use and actual antimicrobial agent use.

Future directions

If passively acquired laboratory data is to contribute to national surveillance of antimicrobial resistance in bacterial pathogens from animals, further study is suggested in the following areas:

1. Diagnostic test performance

Further understanding of the performance of disc diffusion and broth microdilution for bacterial pathogens of interest to national surveillance is needed. In this thesis, the performance of disc diffusion for the two organisms evaluated was reliant on characteristics of the antimicrobial agent, the interpretative criteria used to determine susceptibility, and the accuracy of broth microdilution (as the reference assay). Since these factors change with the bacterial species under evaluation, it is not appropriate to generalise the performance of disc diffusion to other bacteria of interest to national surveillance. Also, the performance of broth microdilution must be evaluated to ensure the quality of MIC data generated for surveillance.

2. Quality of phenotypic data in veterinary diagnostic laboratories

A thorough evaluation of the quality of disc diffusion and MIC susceptibility data as it is generated in veterinary laboratories is needed before passive surveillance can be instituted. This includes the evaluation of recording and reporting of quantitative results, the ease of data retrieval from existing laboratory information systems (LIMS), and the adequacy of laboratory submission forms for epidemiological evaluation. Also, the coverage of isolates derived from bacterial pathogens of interest needs to be quantified.

3. Establishment of veterinary diagnostic laboratory trial 'surveillance sites' in Australia

Consideration should be given to the establishment of a trial of veterinary diagnostic laboratory 'surveillance sites' similar to those used in human antimicrobial resistance surveillance. The trial would ascertain if it is practical for veterinary diagnostic laboratories to adopt standardised protocols for phenotypic assays (broth microdilution and disc diffusion), use stipulated antimicrobial agent panels and consistently record and report susceptibility data for targeted bacterial pathogens, alongside the day-to-day evaluation of clinical submissions. For this to be a success, clinical microbiologists, epidemiologists, and regulators must be able to reach consensus on the most advantageous approach to passive surveillance within diagnostic laboratories.

4. Collection of farm-level antimicrobial use data

Improved survey methods are needed to collect high-quality farm-level data on antimicrobial use. Collection methods must be on-going to monitor trends over time and evaluate the impact of interventions to manage antimicrobial resistance. The antimicrobial use data collected must meet OIE reporting requirements to enable international comparisons. Industry leaders and government must collaborate to identify ways to maximise veterinary and farmer engagement in the collection of antimicrobial data.

5. Effective communication of antimicrobial resistance in animals

Further study is needed to develop simple, interactive communication tools, such as the Antimicrobial agent Use Risk Matrix, which can drive behavioural change among veterinarians and farmers. Understanding factors that influence antimicrobial use by veterinarians and farmers are needed to design the best communication tools to bring about changes in behaviour.

Final Remarks

The research presented in this thesis has identified that for bacterial pathogens, susceptibility data from disc diffusion or broth microdilution generated in veterinary laboratories can contribute to national surveillance. This information, coupled with data from surveys of antimicrobial use at the farm-level, will be of substantial benefit to the development of interventions aimed at containing antimicrobial resistance in animals. Collaboration between veterinary laboratories, farmers, veterinarians, and regulators is essential to ensure the collection of relevant, high-quality data for use in national surveillance.

References

Enoe, C., Georgiadis, M.P., Johnson, W.O., 2000. Estimation of sensitivity and specificity of diagnostic tests and disease prevalence when the true disease state is unknown. Preventive veterinary medicine 45, 61-81.

Green, T.A., Black, C.M., Johnson, R.E., 1998. Evaluation of bias in diagnostic-test sensitivity and specificity estimates computed by discrepant analysis. Journal of clinical microbiology 36, 375-381.

Hombach, M., Zbinden, R., Bottger, E.C., 2013. Standardisation of disk diffusion results for antimicrobial agent susceptibility testing using the sirscan automated zone reader. BMC Microbiology, 13, 225.

Idelevich, E.A., Becker, K., Schmitz, J., Knaack, D., Peters, G., Kock, R., 2016. Evaluation of an automated system for reading and interpreting disk diffusion antimicrobial susceptibility testing of fastidious bacteria. PLoS One 11, e0159183.

Johnson, W.O., Jones, G., Gardner, I.A., 2019. Gold standards are out and Bayes is in: Implementing the cure for imperfect reference tests in diagnostic accuracy studies. Preventive veterinary medicine 167, 113-127.

Meat & Livestock Australia, 2018. Antimicrobial Stewardship Guidelines for the Australian Cattle Feedlot Industry. MLA, Sydney.

Miller, W.C., 2012. Commentary: Reference-test bias in diagnostic-test evaluation: a problem for epidemiologists, too. Epidemiology 23, 83-85.

Murdoch, M., Simon, A.B., Polusny, M.A., Bangerter, A.K., Grill, J.P., Noorbaloochi, S., Partin, M.R., 2014. Impact of different privacy conditions and incentives on survey response rate, participant representativeness, and disclosure of sensitive information: a randomized controlled trial. BMC medical research methodology 14, 90.

OIE, 2017. OIE Annual report on the use of antimicrobial agents intended for use in animals: better understanding of the global situation. 2nd Report.

Pepe, M.S., Janes, H., 2007. Insights into latent class analysis of diagnostic test performance. Biostatistics 8, 474-484.

WHO, 2017. Global antimicrobial resistance surveillance system (GLASS) report: early implementation 2016-2017. World Health Organization Geneva.

Appendix 1

Supplementary Materials for Chapter 2:

Relative Performance of Antimicrobial Susceptibility Assays on Clinical *Escherichia coli* Isolates from Animals

Supplementary table 1 Diagnostic performance estimates of disc diffusion relative to broth microdilution for 994 clinical *Escherichia coli* isolates from animals using CLSI susceptible and resistant breakpoints. DSe, diagnostic sensitivity; DSp, diagnostic specificity; AUC, area under the curve.

	Susceptible breakpoint estimates			Resistant breakpoint estimates		
Antimicrobial	**DSe (95% CI)**	**DSp (95% CI)**	**AUC[a] (95% CI)**	**DSe (95% CI)**	**DSp (95% CI)**	**AUC (95% CI)**
Amoxicillin-clavulanic acid	0.23(0.20, 0.26)	0.97 (0.96, 1.0)	0.82 (0.79, 0.85)	0.79 (0.70, 0.87)	0.99 (0.99, 1.0)	0.98 (0.96, 1.0)
Amikacin	NA	0.99 (0.98, 1.0)	NA	NA	1.0 (0.99, 1.0)	NA
Ampicillin	0.93 (0.90, 0.96)	0.81 (0.77, 0.84)	0.96 (0.94, 0.97)	0.97 (0.94, 0.99)	0.95 (0.94, 0.97)	0.98 (0.96, 0.99)
Cephalothin	0.70 (0.67, 0.73)	0.81 (0.71, 0.89)	0.82 (0.77, 0.88)	0.75 (0.69, 0.81)	0.98 (0.97, 0.99)	0.92 (0.89, 0.95)
Ceftiofur	0.84 (0.76, 0.90)	0.99 (0.98, 0.99)	0.94 (0.91, 0.97)	0.94 (0.87, 0.98)	0.99 (0.98, 1.0)	0.98 (0.96, 1.0)
Ciprofloxacin	0.96 (0.89, 0.99)	1.0 (0.99, 1.0)	0.99 (0.97, 1.0)	0.99 (0.93, 1.0)	1.0 (0.99, 1.0)	1.0 (1.0, 1.0)
Cefovecin	0.67 (0.59, 0.75)	0.96 (0.96, 0.97)	0.87 (0.83, 0.91)	0.88 (0.81, 0.94)	0.99 (0.99, 1.0)	0.97 (0.94, 1.0)
Cefoxitin	0.33 (0.28, 0.40)	1.0(0.99, 1.0)	0.78 (0.74, 0.82)	0.83 (0.73, 0.90)	0.99 (0.99, 1.0)	0.97 (0.95, 1.0)
Gentamicin	0.50 (0.39, 0.60)	0.99 (0.98, 1.0)	0.82 (0.77, 0.87)	0.92 (0.80, 0.98)	1.0 (0.99, 1.0)	0.97 (0.94, 1.0)
Imipenem	NA	0.99 (0.98, 1.0)	NA	NA	1.0 (0.99, 1.0)	NA
Trimethoprim-sulfamethoxazole	0.70 (0.63, 0.76)	0.99 (0.98, 1.0)	0.93 (0.91, 0.96)	0.72 (0.65, 0.79)	0.99 (0.98, 1.0)	0.94 (0.92, 0.97)
Tetracycline	0.93 (0.89, 0.96)	0.98 (0.97, 0.99)	0.97 (0.95, 0.99)	0.95 (0.91, 0.98)	0.99 (0.99, 1.0)	0.98 (0.96, 0.99)

CI, 95% confidence interval (exact).

NA, not available due to insufficient data for the analysis.

Supplementary Table 2 Estimates of likelihood ratio of disc diffusion relative to broth microdilution for 994 clinical *Escherichia coli* isolates using CLSI susceptible and resistant breakpoints. LR^+, likelihood ratio of a positive test result; LR^-, likelihood ratio of a negative test result.

	Susceptible Breakpoint Estimates		**Resistant Breakpoint Estimates**	
Antimicrobial	**LR^+ (95% CI)**	**LR^- (95% CI)**	**LR^+ (95% CI)**	**LR^- (95% CI)**
Amoxicillin-clavulanic acid	15.8 (5.1, 49.0)	0.79 (0.75, 0.82)	118.1 (52.9, 263.7)	0.21 (0.14, 0.30)
Amikacin	5.5 (0.72, 42.3)	0.96 (0.88, 1.1)	NA	NA
Ampicillin	4.8 (4.1, 5.6)	0.09 (0.06, 0.13)	21.0 (15.0, 29.3)	0.03 (0.02, 0.06)
Cephalothin	3.7 (2.4, 5.9)	0.37 (0.32, 0.43)	35.4 (22.0, 57.1)	0.25 (0.20, 0.32)
Ceftiofur	67.3 (37.2, 121.7)	0.16 (0.11, 0.25)	168.4 (70.2, 404.2)	0.06 (0.03, 0.14)
Ciprofloxacin	220.6 (82.9, 587.1)	0.04 (0.01, 0.12)	454.6 (113.8, 1815.4)	0.01 (0, 0.10)
Cefovecin	17.2 (12.1, 24.5)	0.34 (0.27, 0.43)	131.2 (58.9, 292.1)	0.12 (0.07, 0.20)
Cefoxitin	61.8 (22.9, 166.9)	0.67 (0.61, 0.73)	124.9 (56.0, 279.0)	0.18 (0.11, 0.28)
Gentamicin	63.3 (29.5, 135.9)	0.51 (0.42, 0.62)	289.3 (93.2, 898.1)	0.08 (0.03, 0.21)
Imipenem	5.0 (1.5, 16.9)	0.94 (0.87, 1.0)	NA	NA
Trimethoprim-sulfamethoxazole	68.8 (34.3, 137.9)	0.31 (0.25, 0.38)	72.9 (36.4, 146.1)	0.28 (0.22, 0.35)
Tetracycline	53.5 (31.8, 90.1)	0.07 (0.04, 0.12)	154.4 (64.4, 370.1)	0.05 (0.03, 0.09)

CI, 95% confidence interval (exact).

NA, not available due to insufficient data for the analysis.

Supplementary Table 3 Agreement estimates between broth microdilution and disc diffusion for 994 clinical *Escherichia coli* isolates from animals using CLSI *susceptible* breakpoints. BMD, broth microdilution; DD, disc diffusion.

Antimicrobial	**Resistant BMD**	**Resistant DD**	**McNemars p-value**[†]	**Observed agreement (95% CI)**	**Positive agreement (95% CI)**	**Negative agreement (95% CI)**	**PABAK (95% CI)**
Amoxicillin-clavulanic acid	0.79	0.18	<0.001*	0.39 (0.36, 0.42)	0.37 (0.34, 0.40)	0.41 (0.36, 0.44)	NA
Amikacin	0.02	0.01	0.02*	0.97 (0.96, 0.98)	0 (0, 0.21)	0.99 (0.98, 0.99)	0.94 (0.92, 0.96)
Ampicillin	0.35	0.45	<0.001*	0.85 (0.83, 0.87)	0.81 (0.78, 0.84)	0.87 (0.85, 0.89)	0.70 (0.65, 0.74)
Cephalothin	0.92	0.66	<0.001*	0.71 (0.67, 0.73)	0.81 (0.79, 0.83)	0.31 (0.26, 0.34)	0.41 (0.35, 0.47)
Ceftiofur	0.11	0.11	0.20	0.97 (0.96, 0.98)	0.87 (0.81, 0.91)	0.98 (0.98,0.99)	0.94 (0.92, 0.96)
Ciprofloxacin	0.08	0.08	0.73	0.99 (0.97, 1.0)	0.95 (0.91, 0.98)	1.0 (0.99, 1.0)	0.99 (0.98, 1.0)
Cefovecin	0.15	0.14	0.08	0.92 (0.90, 0.93)	0.71 (0.66, 0.76)	0.95 (0.94, 0.96)	0.84 (0.80, 0.87)
Cefoxitin	0.25	0.09	<0.001*	0.83 (0.80, 085)	0.49 (0.44, 0.55)	0.90 (0.88, 0.91)	0.65 (0.61, 0.70)
Gentamicin	0.10	0.06	<0.001*	0.94 (0.93, 0.96)	0.63 (0.55, 0.71)	0.97 (0.96, 0.98)	0.89 (0.86, 0.92)
Imipenem	0.04	0.02	<0.001*	0.95 (0.93, 0.96)	0.1 (0, 0.21)	0.97 (0.96, 0.98)	0.89 (0.82, 0.89)
Trimethoprim-sulfamethoxazole	0.21	0.15	<0.001*	0.93 (0.91, 0.95)	0.80 (0.76, 0.84)	0.96 (0.95, 0.97)	0.86 (0.82, 0.89)
Tetracycline	0.19	0.19	0.85	0.97 (0.96, 0.98)	0.93 (0.90, 0.95)	0.98 (0.98, 0.99)	0.95 (0.93, 0.97)

CI, Confidence interval (95% exact).

NA, not available due to insufficient data for the analysis.

* Represents a statistically significant mid-*p* McNemar's chi-square test ($p < 0.05$).

Supplementary Table 4 Agreement estimates between broth microdilution and disc diffusion for 994 clinical *Escherichia coli* isolates from animals using CLSI *resistant* breakpoints. BMD, broth microdilution; DD, disc diffusion.

Antimicrobial	Resistant BMD	Resistant DD	McNemars p-value†	Observed agreement (95% CI)	Positive agreement (95% CI)	Negative agreement (95% CI)	PABAK (95% CI)
Amoxicillin-clavulanic acid	0.10	0.09	<0.001*	0.97 (0.96, 0.98)	0.86 (0.80, 0.90)	0.98 (0.98, 0.99)	0.95 (0.93, 0.97)
Amikacin	0.02	0.02	0.63	1.0 (0.99, 1.0)	0 (0, 0.60)	1.0 (0.99, 1.0)	NA
Ampicillin	0.28	0.30	<0.001*	0.96 (0.94, 0.97)	0.93 (0.90, 0.95)	0.97 (0.96, 0.98)	0.92 (0.89, 0.94)
Cephalothin	0.20	0.17	<0.001*	0.94 (0.92, 0.95)	0.82 (0.78, 0.86)	0.96 (0.95, 0.97)	0.87 (0.84, 0.90)
Ceftiofur	0.10	0.10	0.77	0.99 (0.98, 0.99)	0.94 (0.90, 0.97)	0.99 (0.99, 1.0)	0.98 (0.97, 0.99)
Ciprofloxacin	0.07	0.07	0.63	1.0 (0.99, 1.0)	0.98 (0.94, 1.0)	1.0 (0.99, 1.0)	0.99 (0.99, 1.0)
Cefovecin	0.10	0.10	0.17	0.98 (0.97, 0.99)	0.91 (0.86, 0.95)	0.99 (0.98, 1.0)	0.97 (0.94, 0.98)
Cefoxitin	0.09	0.08	0.05*	0.98 (0.97, 0.99)	0.87 (0.81, 0.92)	0.99 (0.98, 1.0)	0.96 (0.94, 0.98)
Gentamicin	0.05	0.05	0.73	0.99 (0.99, 1.0)	0.93 (0.86, 0.97)	1.0 (0.99, 1.0)	0.99 (0.98, 1.0)
Imipenem	0	0	0.2	1.0 (0.99, 1.0)	0 (0, 0.52)	1.0 (0.99, 1.0)	0.99 (0.98, 1.0)
Trimethoprim-sulfamethoxazole	0.19	0.15	<0.001*	0.94 (0.92, 0.95)	0.82 (0.77, 0.86)	0.96 (0.95, 0.97)	0.88 (0.85, 0.91)
Tetracycline	0.18	0.18	0.30	0.99 (0.99, 1.0)	0.96 (0.94, 0.98)	0.99 (0.98, 1.0)	0.97 (0.96, 0.99)

CI, Confidence interval (95% exact).

NA, not available due to insufficient data for the analysis.

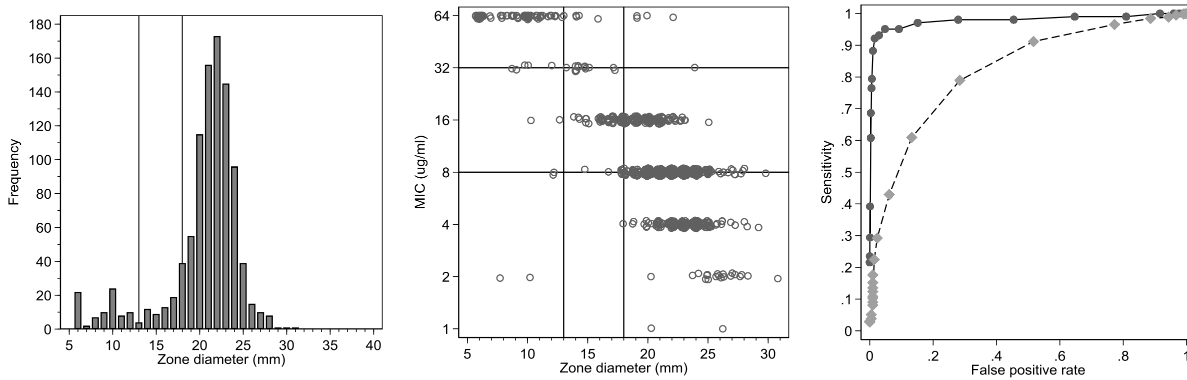
* Represents a statistically significant mid-*p* McNemar's chi-square test ($p < 0.05$).

Supplementary Table 5 Estimates of accuracy of disc diffusion relative to broth microdilution for 994 clinical *Escherichia coli* isolates from animals using zone diameter interpretative criteria produced from the dBETS program. DSE, diagnostic sensitivity; DSp, diagnostic specificity; ZD, zone diameter.

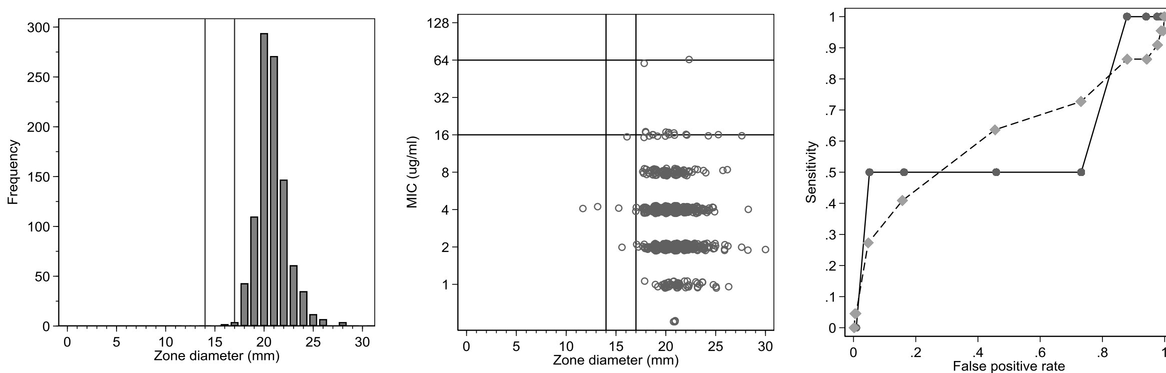
	dBETS Susceptible Breakpoint Estimates				dBETS Resistant Breakpoint Estimates			
Antimicrobial	**ZD susceptible breakpoint (mm)**	**DSe (95% CI)**	**DSp (95% CI)**	**Observed agreement**	**ZD resistant breakpoint (mm)**	**DSe (95% CI)**	**DSp (95% CI)**	**Observed agreement**
Amoxicillin-clavulanic acid	21	0.61 (0.57, 0.64)	0.87 (0.81, 0.91)	0.66 (0.63, 0.69)	15	0.92 (0.85, 0.97)	0.98 (0.97, 0.99)	0.98 (0.97, 0.99)
Amikacin	16	NA	1.0 (0.99, 1.0)	0.97 (0.96, 0.98)	12	NA	1.0 (0.99, 1.0)	1.0 (0.99, 1.0)
Ampicillin	11	0.80 (0.76, 0.85)	0.98 (0.96, 0.99)	0.92 (0.90, 0.93)	7	0.96 (0.94, 0.98)	0.98 (0.97, 0.99)	0.97 (0.96, 0.98)
Cephalothin	18	0.70 (0.67, 0.73)	0.81 (0.71, 0.89)	0.71 (0.68, 0.73)	13	0.68 (0.61, 0.74)	0.99 (0.98, 1.0)	0.93 (0.91, 0.94)
Ceftiofur	22	0.86 (0.78, 0.92)	0.98 (0.97, 0.99)	0.97 (0.95, 0.98)	18	0.96 (0.90, 0.99)	0.99 (0.97, 1.0)	0.99 (0.98, 1.0)
Ciprofloxacin	18	0.96 (0.89, 0.99)	1.0 (0.99, 1.0)	1.0 (0.99, 1.0)	11	0.90 (0.80, 0.96)	1.0 (0.99, 1.0)	0.99 (0.98, 1.0)
Cefovecin	23	0.67 (0.60, 0.75)	0.96 (0.95, 0.97)	0.92 (0.90, 0.93)	19	0.88 (0.81, 0.94)	0.99 (0.99, 1.0)	0.98 (0.97, 0.99)
Cefoxitin	22	0.43 (0.37, 0.50)	0.97 (0.96, 0.98)	0.83 (0.81, 0.86)	18	0.91 (0.83, 0.96)	0.99 (0.98, 1.0)	0.98 (0.97, 0.99)
Gentamicin	16	0.50 (0.39, 0.60)	0.99 (0.98, 1.0)	0.94 (0.93, 0.96)	12	0.92 (0.80, 0.98)	1.0 (0.99, 1.0)	0.99 (0.98, 1.0)
Imipenem	23	NA	0.99 (0.98, 0.99)	0.95 (0.93, 0.96)	15	NA	1.0 (0.99, 1.0)	1.0 (0.99, 1.0)
Trimethoprim-sulfamethoxazole	25	0.87 (0.82, 0.92)	0.86 (0.83, 0.88)	0.86 (0.84, 0.88)	21	0.79 (0.72, 0.84)	0.98 (0.96, 0.99)	0.94 (0.92, 0.96)
Tetracycline	18	0.93 (0.88, 0.96)	0.99 (0.98, 0.99)	0.97 (0.96, 0.98)	13	0.95 (0.91, 0.98)	0.99 (0.99, 1.0)	0.98 (0.97, 0.99)

NA, not available due to insufficient data.

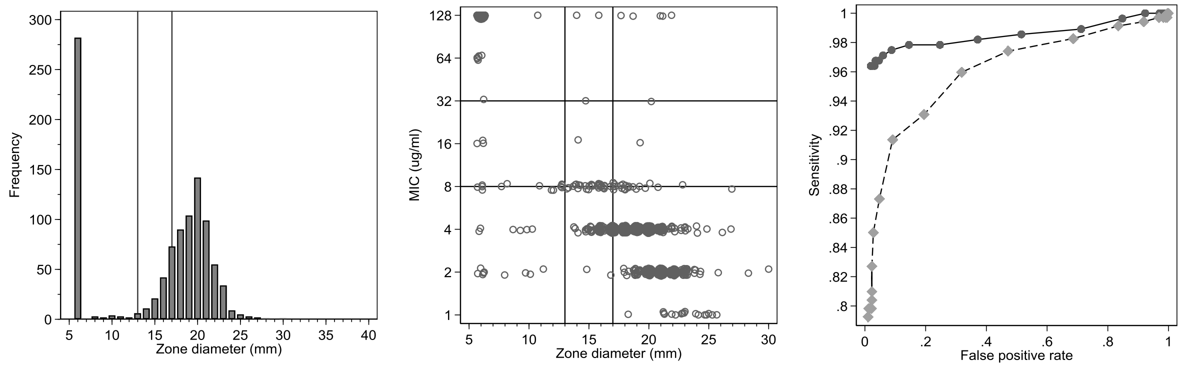
Supplementary Figures



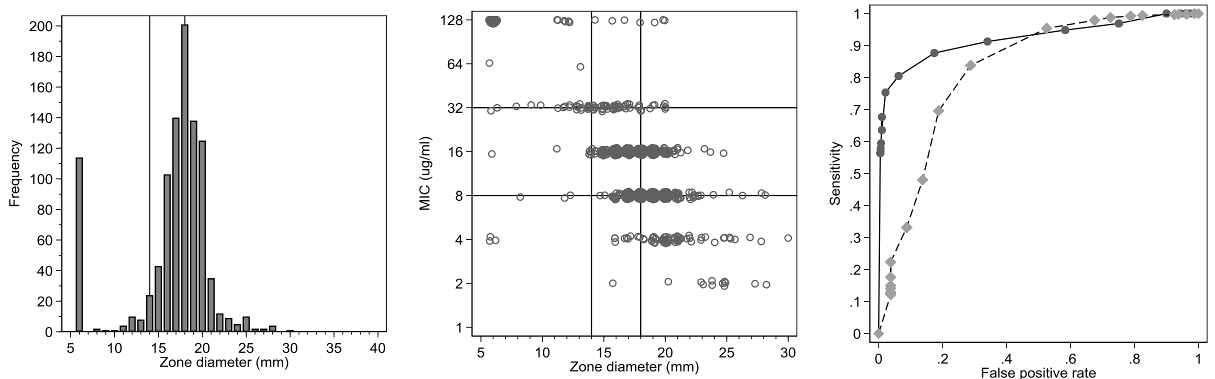
Supplementary Figure 1. Amoxicillin-clavulanic acid - performance of disc diffusion relative to broth microdilution for clinical *E. coli* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on $\log_2$ scale), and (iii) two-graph ROC curve. See text for interpretation of plots.



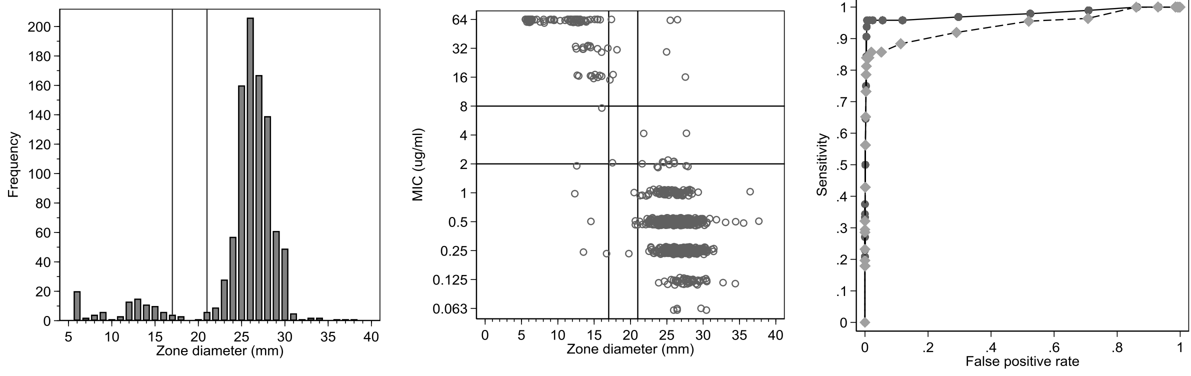
Supplementary Figure 2. Amikacin - performance of disc diffusion relative to broth microdilution for clinical *E. coli* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on $\log_2$ scale), and (iii) two-graph ROC curve. See text for interpretation of plots.



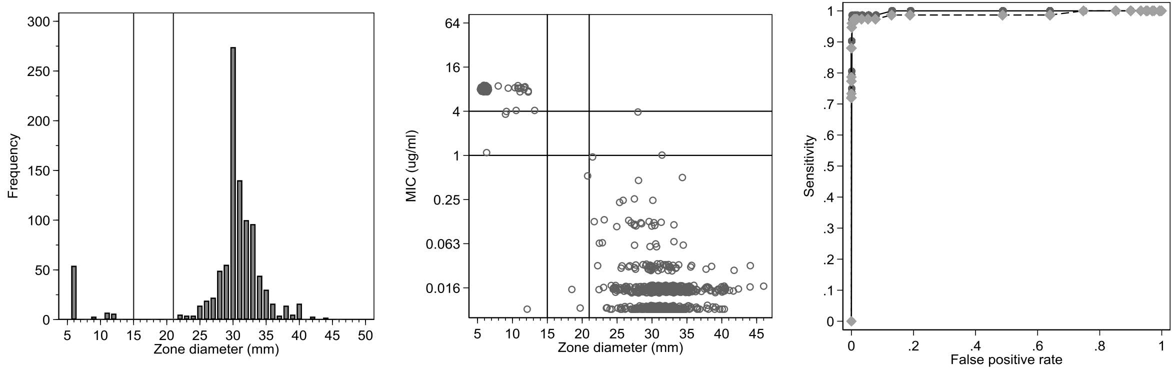
Supplementary Figure 3. Ampicillin - performance of disc diffusion relative to broth microdilution for clinical *E. coli* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on $\log_2$ scale), and (iii) two-graph ROC curve. See text for interpretation of plots.



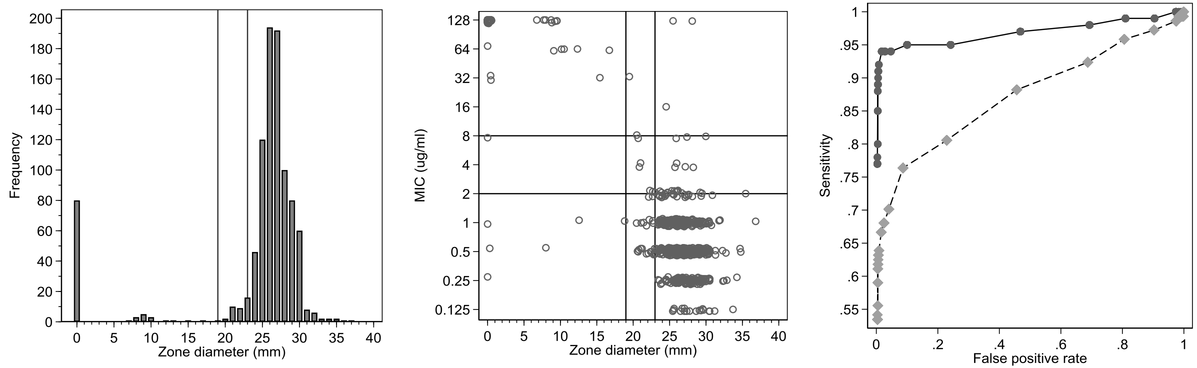
Supplementary Figure 4. Cephalothin - performance of disc diffusion relative to broth microdilution for clinical *E. coli* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on $\log_2$ scale), and (iii) two-graph ROC curve. See text for interpretation of plots.



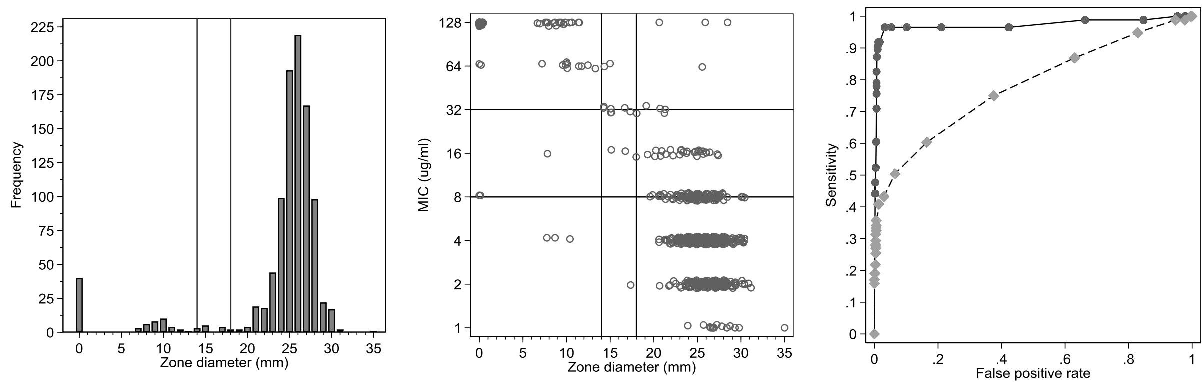
Supplementary Figure 5. Ceftiofur - performance of disc diffusion relative to broth microdilution for clinical *E. coli* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on $\log_2$ scale), and (iii) two-graph ROC curve. See text for interpretation of plots.



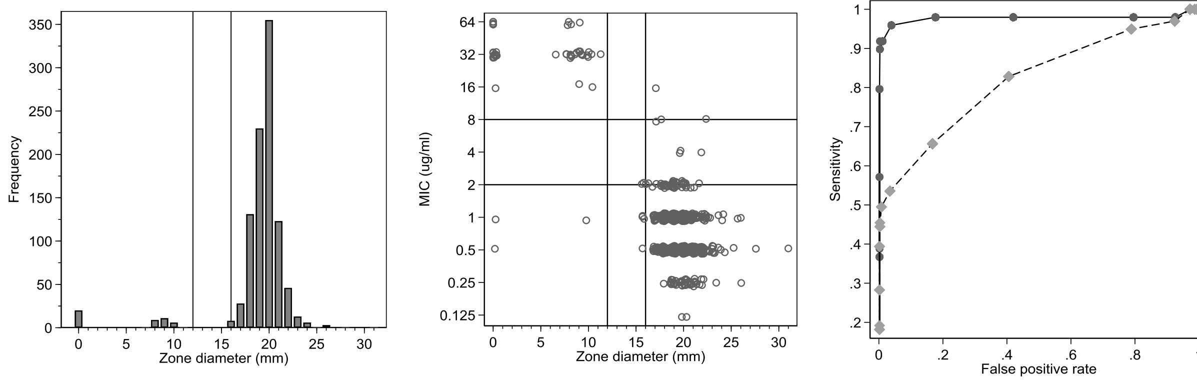
Supplementary Figure 6. Ciprofloxacin - performance of disc diffusion relative to broth microdilution for clinical *E. coli* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on $\log_2$ scale), and (iii) two-graph ROC curve. See text for interpretation of plots.



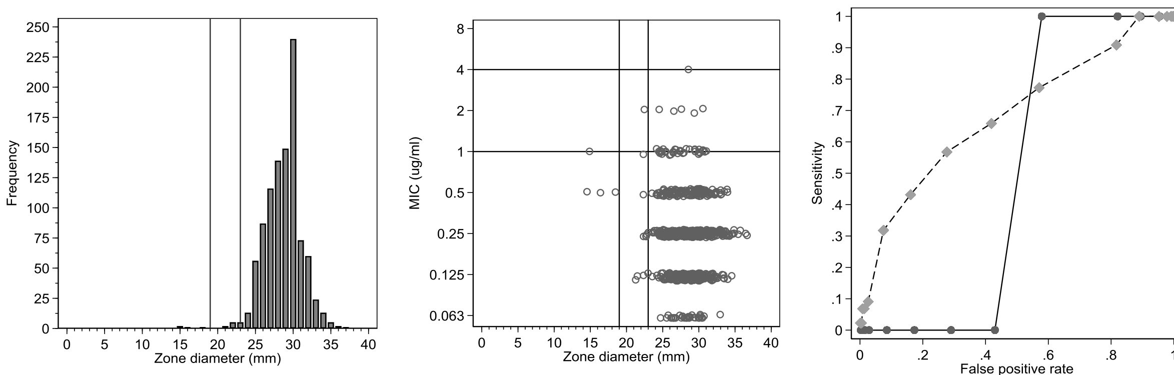
Supplementary Figure 7. Cefovecin - performance of disc diffusion relative to broth microdilution for clinical *E. coli* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on log_2 scale), and (iii) two-graph ROC curve. See text for interpretation of plots.



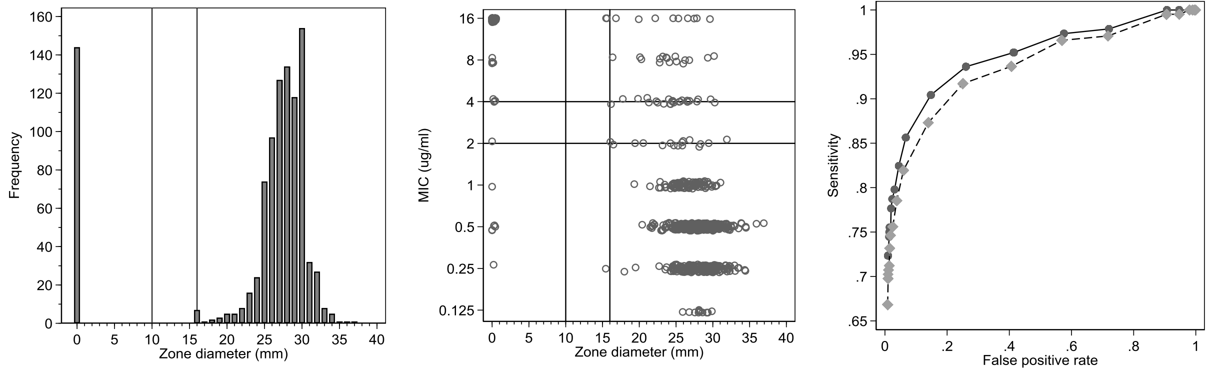
Supplementary Figure 8. Cefoxitin - performance of disc diffusion relative to broth microdilution for clinical *E. coli* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on log_2 scale), and (iii) two-graph ROC curve. See text for interpretation of plots.



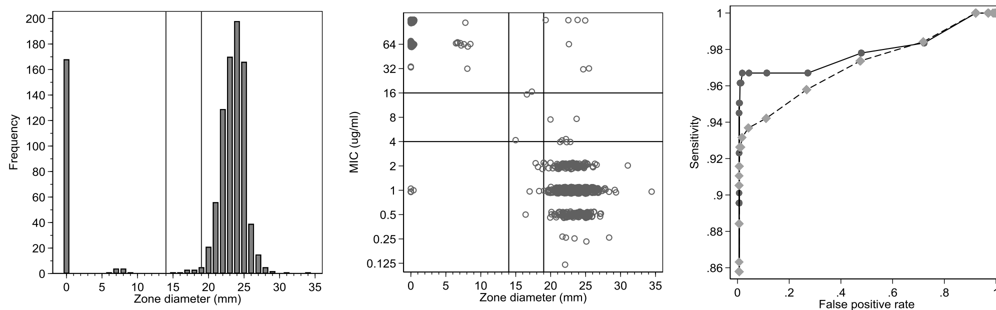
Supplementary Figure 9. Gentamicin - performance of disc diffusion relative to broth microdilution for clinical *E. coli* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on $\log_2$ scale), and (iii) two-graph ROC curve. See text for interpretation of plots.



Supplementary Figure 10. Imipenem - performance of disc diffusion relative to broth microdilution for clinical *E. coli* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on $\log_2$ scale), and (iii) two-graph ROC curve. See text for interpretation of plots.



Supplementary Figure 11. Trimethoprim-sulfamethoxazole - performance of disc diffusion relative to broth microdilution for clinical *E. coli* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on $\log_2$ scale), and (iii) two-graph ROC curve. See text for interpretation of plots.



Supplementary Figure 12. Tetracycline - performance of disc diffusion relative to broth microdilution for clinical *E. coli* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on $\log_2$ scale), and (iii) two-graph ROC curve. See text for interpretation of plots.

Appendix 2

Supplementary material from Chapter 3:

Diagnostic accuracy of phenotypic assays for determining antimicrobial resistance status in *Staphylococcus pseudintermedius* isolates from canine clinical cases

Supplementary Table 1. Agreement estimates between broth microdilution and disc diffusion for 614 canine *Staphylococcus pseudintermedius* isolates from clinical cases. Clinical Laboratory Standards Institute (CLSI) **susceptible** breakpoints were used to dichotomise MIC and zone diameter values. Exact 95% confidence intervals are given. BMD, broth microdilution; DD, disc diffusion; PABAK, prevalence-adjusted bias-adjusted kappa.

Antimicrobial	% BMD resistant	% DD resistant	McNemars mid *p*-value	% Observed agreement	% Positive agreement	% Negative Agreement	PABAK
Amoxicillin-clavulanic acid	10.7 (8.4, 13.5)	5.7 (4.0, 7.8)	<0.001*	94.6 (92.5, 96.3)	67.3 (57.3, 76.3)	97.1 (95.9, 98.0)	0.89 (0.86, 0.93)
Cefovecin	13.5 (10.9, 16.5)	10.6 (8.3, 13.3)	<0.001*	95.4 (93.5, 97.6)	81.1 (73.8, 87.1)	97.4 (96.3, 98.3)	0.91 (0.90, 0.96)
Cefoxitin	2.3 (1.3, 3.8)	1.5 (0.7, 2.8)	0.18	97.9 (96.4, 98.9)	43.5 (23.2, 65.5)	98.2 (98.2, 99.4)	0.96 (0.94, 0.98)
Cephalothin	7.3 (5.4, 9.7)	5.2 (3.6, 7.3)	<0.001*	97.2 (95.6, 98.4)	77.9 (67.0, 86.6)	98.5 (97.6, 99.1)	0.95 (0.92, 0.97)
Chloramphenicol	6.5 (4.7, 8.8)	5.5 (3.9, 7.7)	0.02*	99.0 (97.8, 99.6)	91.9 (83.2,97.0)	99.5 (98.9, 99.8)	0.98 (0.97, 1.0)
Ciprofloxacin	9.0 (6.8, 11.5)	8.3 (6.3, 10.8)	0.13	99.0 (97.9, 99.6)	94.3 (88.1, 97.9)	99.5 (98.8, 99.8)	0.98 (0.97, 1.0)
Clindamycin	13.4 (10.8, 16.3)	13.8 (11.2, 16.8)	0.22	99.2 (98.1, 99.7)	97.0 (93.2, 99.0)	99.5 (98.9, 99.9)	0.98 (0.97, 1.0)
Oxacillin	12.9 (10.3, 14.8)	11.4 (9.0, 14.2)	<0.001*	98.5 (97.2, 99.3)	94.0 (88.8, 97.2)	99.2 (98.4, 99.6)	0.97 (0.95, 0.99)
Rifampicin	0.8 (0.3, 18.9)	1.1 (4.6, 2.3)	0.37	99.4 (98.3, 99.8)	66.7 (34.9, 90.1)	99.7 (99.2, 99.9)	0.99 (0.97, 1.0)
Tetracycline	22.3 (19.1, 25.8)	22.8 (19.9, 26.3)	0.31	96.3 (94.4, 97.6)	91.8 (87.9, 94.7)	97.6 (96.4, 98.5)	0.93 (0.90, 0.96)

* Significant mid-*p* McNemar's chi-square test ($p<0.05$).

Supplementary Table 2. Agreement estimates between broth microdilution and disc diffusion for 614 canine *Staphylococcus pseudintermedius* isolates from clinical cases. Clinical Laboratory Standards Institute (CLSI) **resistant** breakpoints were used to dichotomise MIC and zone diameter values. Exact 95% confidence intervals for estimates are given. BMD, broth microdilution; DD, disc diffusion; PABAK, prevalence-adjusted bias-adjusted kappa.

Antimicrobial	% BMD resistant	% DD resistant	McNemars mid *p*-value	% Observed agreement	% Positive Agreement	% Negative Agreement	PABAK
Amoxicillin-clavulanic acid	10.7 (8.4, 13.5)	5.7 (4.0, 7.8)	<0.001*	94.6 (92.5, 96.3)	67.3 (57.3, 76.3)	97.1 (95.9, 98.0)	0.89 (0.86, 0.93)
Cefovecin	10.3 (8.0, 12.9)	10.1 (7.8, 12.8)	0.81	97.2 (95.6, 98.4)	86.4 (79.1, 91.9)	98.5 (97.5, 99.1)	0.95 (0.92, 0.97)
Cefoxitin	2.3 (1.3, 3.8)	1.5 (6.7, 2.8)	0.18	97.9 (96.4, 98.9)	43.5 (23.2, 65.5)	98.9 (98.2, 99.4)	0.96 (0.94, 0.98)
Cephalothin	6.4 (4.6, 8.6)	2.9 (1.8, 4.6)	<0.001*	96.3 (94.4, 97.6)	59.7 (45.8, 72.4)	98.0 (97.1, 98.8)	0.93 (0.90, 0.96)
Chloramphenicol	5.9 (4.1, 8.0)	5.5 (3.9, 7.7)	0.25	99.7 (98.8, 100)	97.1 (90.1, 99.7)	99.8 (99.4, 100)	0.99 (0.98, 1.0)
Ciprofloxacin	8.3 (6.3, 10.8)	7.5 (5.5, 9.9)	0.07	98.9 (97.7, 99.5)	92.8 (85.7, 97.1)	99.4 (98.7, 99.8)	0.98 (0.96, 0.99)
Clindamycin	12.9 (10.3, 15.8)	10.6 (8.3, 13.3)	<0.001*	97.4 (95.8, 98.5)	88.9 (82.6, 93.5)	98.5 (97.6, 99.2)	0.95 (0.92, 0.97)
Oxacillin	12.9 (10.3, 15.8)	11.4 (9.0, 14.2)	<0.001*	98.5 (97.2, 99.3)	94.0 (88.8, 97.2)	99.2 (98.4, 99.6)	0.97 (0.95, 0.99)
Rifampicin	0.8 (2.6, 1.9)	0.8 (2.6, 18.9)	0.63	99.4 (98.3, 99.8)	60.0 (26.2, 87.8)	99.7 (99.2, 99.9)	0.99 (0.97, 1.0)
Tetracycline	22.0 (18.8, 25.5)	22.8 (19.5, 26.3)	0.29	96.6 (94.8, 97.9)	92.4 (88.6, 95.2)	97.8 (96.7, 98.6)	0.93 (0.90, 0.96)

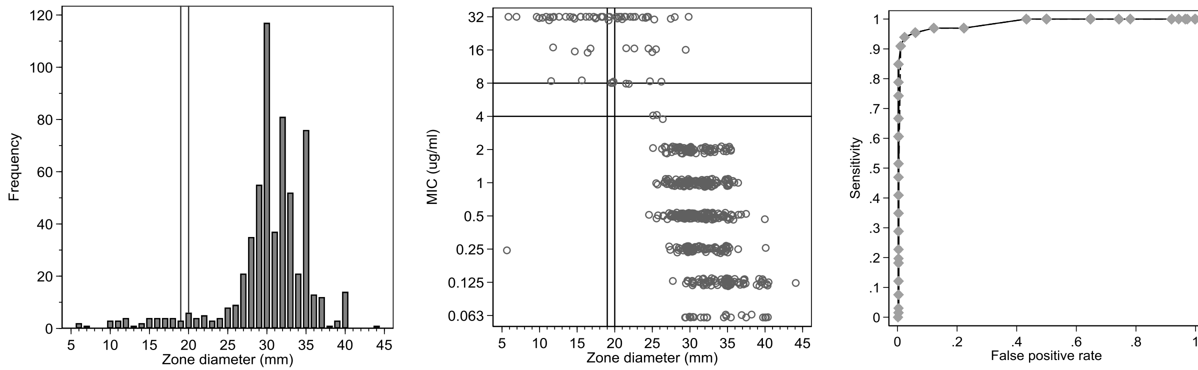
* Significant mid-*p* McNemar's chi-square test ($p<0.05$).

Supplementary Table 3. Molecular ecology of nine *Staphylococcus pseudintermedius* isolates identified as phenotypically susceptible to oxacillin by disc diffusion or broth microdilution in the presence of the *mecA* gene.

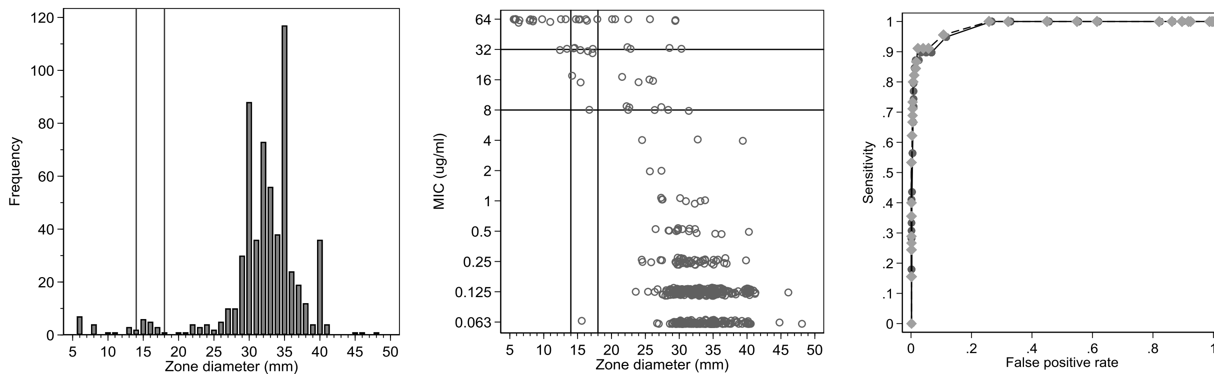
Isolate Identity	Oxacillin MIC (µg/ml)	Oxacillin zone diameter (mm)	Phenotypic Resistance†	MLST	Genotypic resistance
N13/4/19	0.5	20	CVN, OXA	ST539	aac(6')-aph(2"), dfrG, blaZ, mecA
N13/4/59	0.125	29	CLI, OXA	ST498	aac(6')-aph(2"), aadD-like, ant(6)-Ia,aph(3')-III, erm(B)-like, erm(C), blaZ, blaZ-like, mecA
N13/1/616	0.25	19	AMC, CVN, OXA	ST547	blaZ, mecA
N13/1/627	0.5	21	CLI, OXA	ST498	ant(6)-Ia,aph(3')-III, erm(B)-like, blaZ, blaZ-like, mecA
Q13/1/200	0.25	23	TET	Unknown ST	ant(6)-Ia,aph(3')-III, tet(M), blaZ, mecA
V13/2/299	1.0	19	TET	ST71	aac(6')-aph(2"), ant(6)-Ia, aph(3')-III, erm(B)-like, dfrG, blaZ, mecA
V13/2/63	0.5	20	TET	Unknown ST	tet(M)-like, blaZ-like, mecA
V13/2/16	0.5	19	OXA	ST544	blaZ-like, mecA
W13/1/4	0.5	20	CHL, CIP, CLI, OXA, TET	ST45	aac(6')-aph(2"), ant(6)-Ia, aph(3')-III-like, erm(B), dfrG, tet(M), cat_{pC221}-like, blaZ, mecA-like

† Phenotypic resistance is based on Clinical and Laboratory Standards Institute interpretation criteria for disc diffusion and broth microdilution. An isolate underwent whole-genome sequencing if it was oxacillin-susceptible on either disc diffusion or broth microdilution and *mecA* positive on PCR.

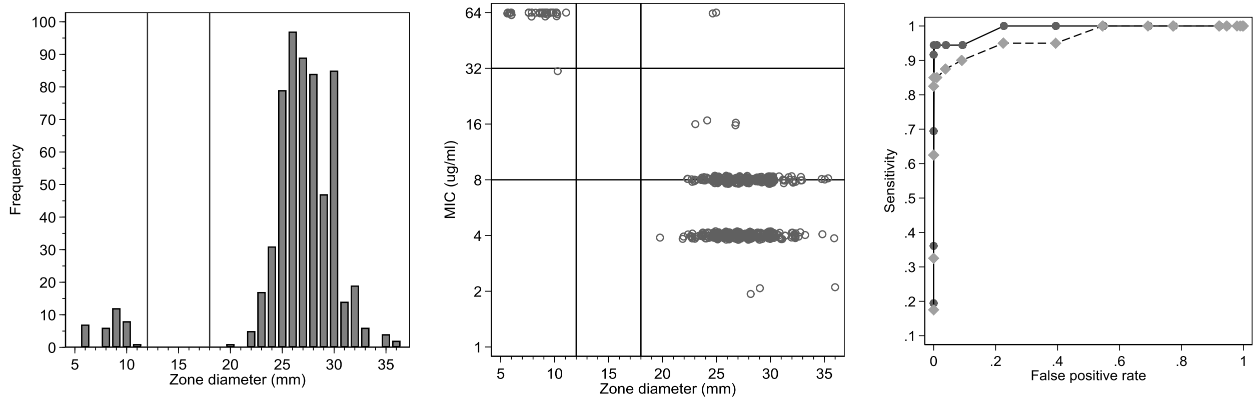
Supplementary Figures



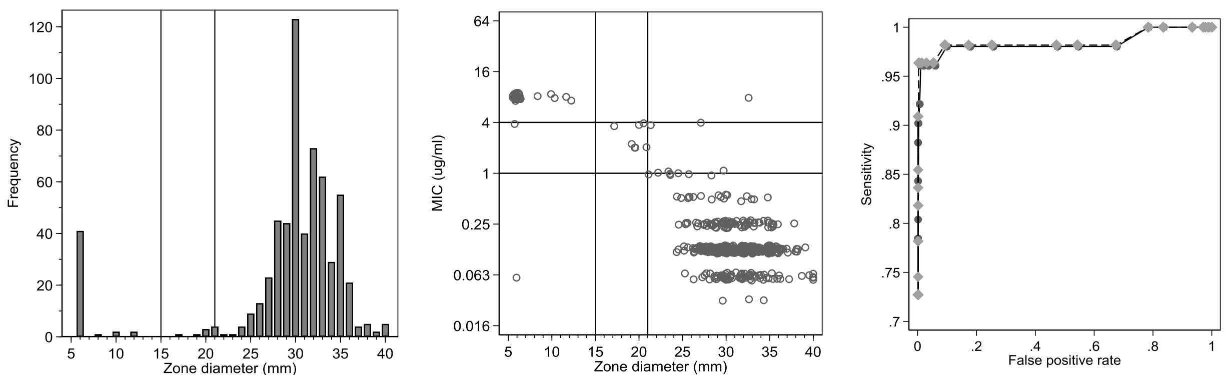
Supplementary figure 1. Amoxicillin-clavulanic acid - performance of disc diffusion relative to broth microdilution for clinical *Staphylococcus pseudintermedius* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on log_2 scale), and (iii) two-graph ROC plot.



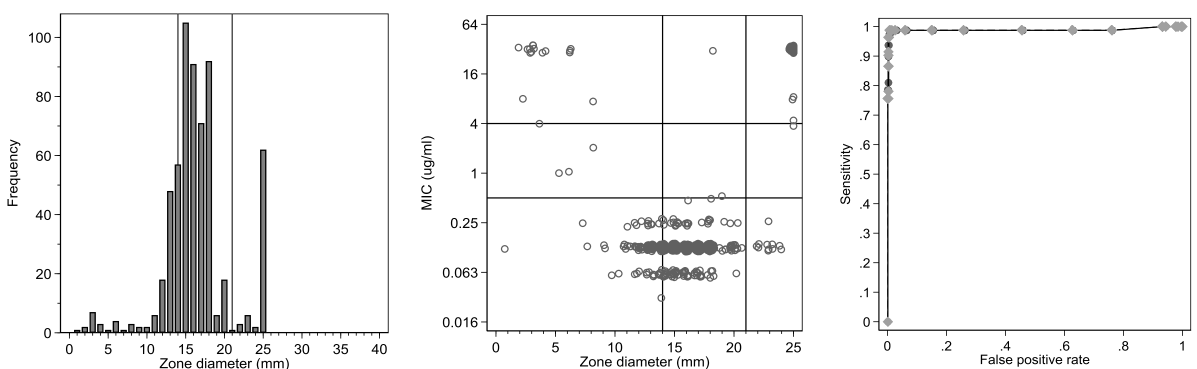
Supplementary figure 2. Cephalothin - performance of disc diffusion relative to broth microdilution for clinical *Staphylococcus pseudintermedius* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on log_2 scale), and (iii) two-graph ROC plot.



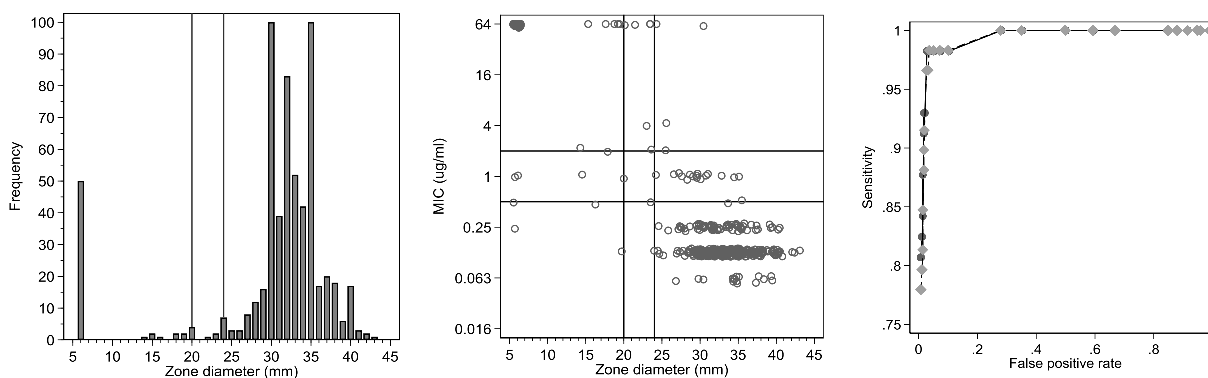
Supplementary figure 3. Chloramphenicol - performance of disc diffusion relative to broth microdilution for clinical *Staphylococcus pseudintermedius* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on log_2 scale), and (iii) two-graph ROC plot.



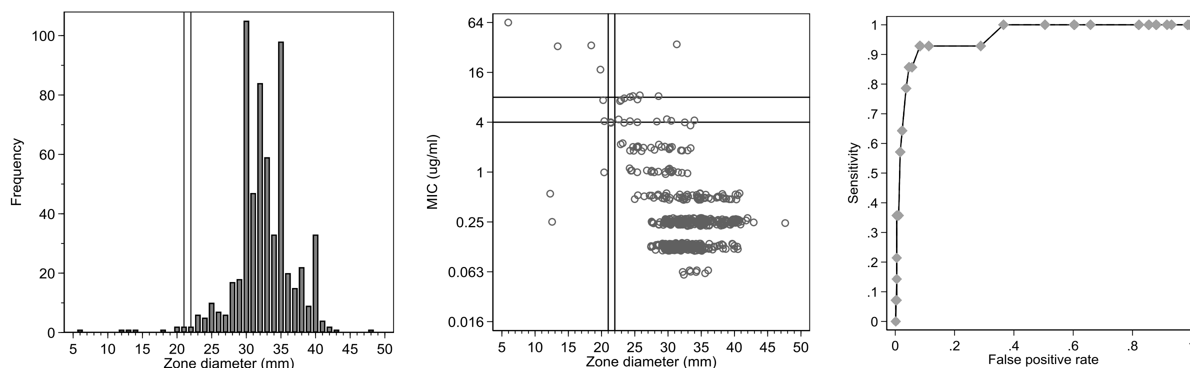
Supplementary figure 4. Ciprofloxacin - performance of disc diffusion relative to broth microdilution for clinical *Staphylococcus pseudintermedius* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on log_2 scale), and (iii) two-graph ROC plot.



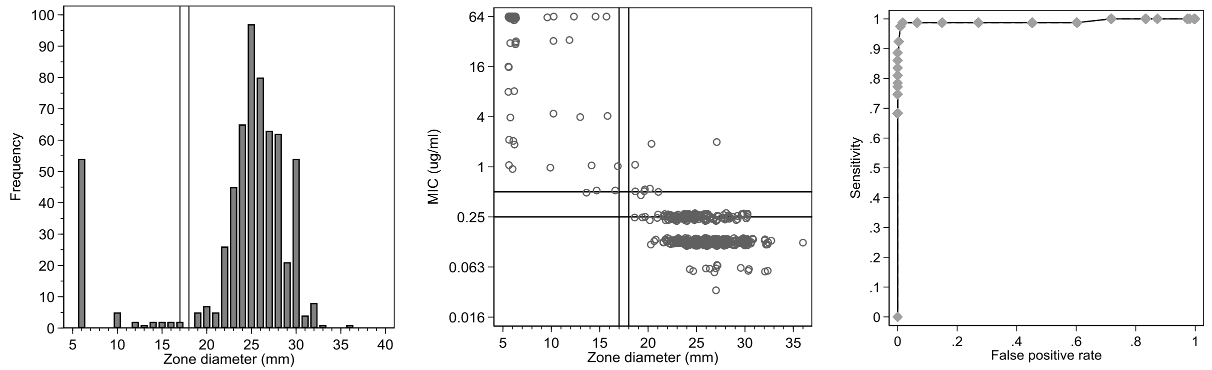
Supplementary figure 5. Clindamycin - performance of disc diffusion relative to broth microdilution for clinical *Staphylococcus pseudintermedius* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on $\log_2$ scale), and (iii) two-graph ROC plot.



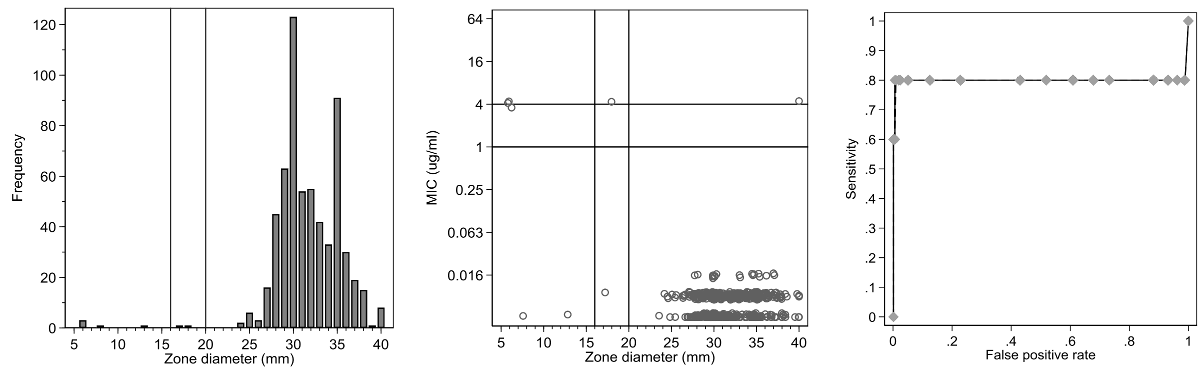
Supplementary figure 6. Cefovecin - performance of disc diffusion relative to broth microdilution for clinical *Staphylococcus pseudintermedius* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on $\log_2$ scale), and (iii) two-graph ROC plot.



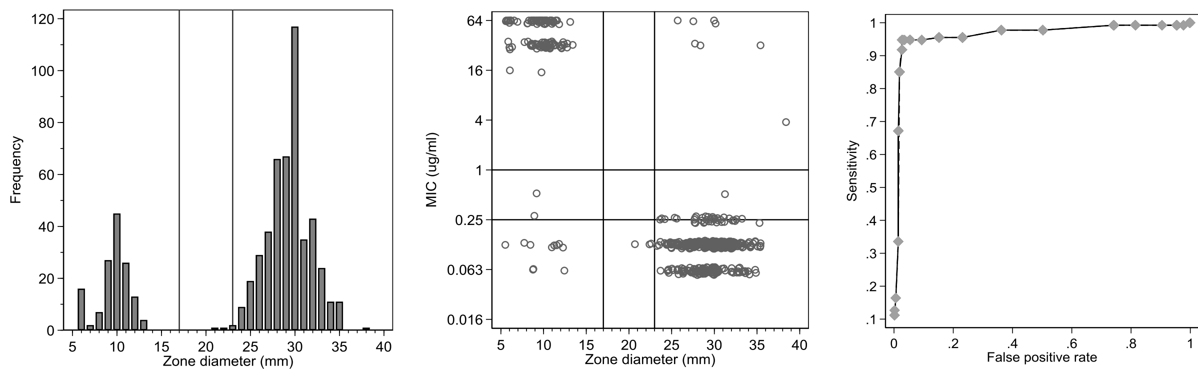
Supplementary figure 7. Cefoxitin - performance of disc diffusion relative to broth microdilution for clinical *Staphylococcus pseudintermedius* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on $\log_2$ scale), and (iii) two-graph ROC plot.



Supplementary figure 8. Oxacillin - performance of disc diffusion relative to broth microdilution for clinical *Staphylococcus pseudintermedius* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on $\log_2$ scale), and (iii) two-graph ROC plot.



Supplementary figure 9. Rifampicin - performance of disc diffusion relative to broth microdilution for clinical *Staphylococcus pseudintermedius* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on $\log_2$ scale), and (iii) two-graph ROC plot.



Supplementary figure 10. Tetracycline - performance of disc diffusion relative to broth microdilution for clinical *Staphylococcus pseudintermedius* isolates from animals (i) distribution of zone diameter results (ii) scatterplot of zone diameter (mm) and MIC (µg/ml, depicted on log_2 scale), and (iii) two-graph ROC plot.

Appendix 3

Supplementary material from Chapter 4:

Intra- and inter-laboratory agreement of the disc diffusion assay for assessing antimicrobial susceptibility of porcine *Escherichia coli*

Supplementary Table 1. Identification of twenty porcine *Escherichia coli* isolates included in an intra- and inter-laboratory agreement study evaluating the performance of the disc diffusion assay in veterinary laboratories.

Isolate identification used in study	Actual isolate identification[a]
1	N13/2/29
2	Q13/4/90
3	V13/7/1
4	N13/2/2
5	Q13/4/97
6	NT/13/1/3
7	V13/5/6
8	W13/2/21
9	V13/5/58
10	V13/5/69
11	V13/5/11
12	Q13/4/54
13	N13/2/7
14	V13/5/48
15	V13/5/50
16	ETEC-S90
17	N13/2/25
18	Q13/4/59
19	Q13/4/134
20	ATCC 25922

a Antimicrobial Resistance and Infectious Diseases Laboratory, Murdoch University

Supplementary Table 2. Descriptive statistics for an intra- and inter-laboratory evaluation of the disc diffusion assay by seven laboratories. Five zone diameter measurements for six antimicrobial agents on 20 *Escherichia coli* isolates was reported by each laboratory on five occasions, representing 35 zone diameter measurements per isolate/ antimicrobial combination.

	Mean/ median/ standard deviation/ minimum-maximum					
Isolate	**Ampicillin**	**Ceftiofur**	**Chloramphenicol**	**Gentamicin**	**Tetracycline**	**Trimethoprim-sulpha**
1	16.6/ 20/ 2.2/ 10-22	26.4/ 26/ 1.7/ 24-30	25.7/ 26/ 1.3/21-28	20/ 20/ 2.0/ 17-29	6.1/ 6/ 0.3/ 6-7	28.2/ 28/ 1.7/ 26-33
2	6.4/ 6/ 2.4/ 6-20	26.4/ 26/ 2.3/ 21-32	6.4/ 6/ 1.7/ 6-15	12.4/ 12/ 3.2/ 6-24	6.1/ 6/ 0.5/ 6-8	6/ 6/ 0/ 6-6
3	6.3/ 6/ 1.9/ 6-17	25.7/ 26/ 1.9/ 22-30	6.6/ 6/ 3.2/ 6-25	9.1/ 9/ 3.4/ 6-22	6/ 6/ 0/ 6-6	7.0/ 6/ 4.3/ 6-28
4	6/ 6/ 0/ 6-6	26.3/ 27/ 3.1/ 10-30	6.1/ 6/ 0.5/ 6-9	8.3/ 8/ 1.3/ 6-10	6/ 6/ 0/ 6-6	6/ 6/ 0/ 6-6
5	16.9/ 18/ 3.1/ 6-22	25.8/ 26/ 1.7/ 20-30	25.7/ 26/ 1.7/ 23-30	20.7/ 21/ 1.3/ 18-23	6.0/ 6/ 0.2/ 6-7	16.7/ 18/ 4.1/ 6-21
6	14.7/ 15/ 2.7/ 6-18	24.9/ 25/ 2.2/ 16-29	21.5/ 22/ 3.5/ 6-25	19.5/ 19/ 1.9/ 15-26	21.3/ 22/ 3.3/ 6-25	25.8/ 26/ 4.0/ 6-31
7	6/ 6/ 0/ 6-6	27.5/ 28/ 3.0/ 15-32	7.1/ 7/ 1.0/ 6-9	10.5/ 10/ 1.5/ 6-14	6.3/ 6/ 0.6/ 6-8	6/ 6/ 0/ 6-6
8	6/ 6/ 0/ 6-6	25.9/ 26/ 2.2/ 22-33	18.7/ 19/ 2.7/ 6-23	9/ 9/ 1.3/ 6-11	6.1/ 6/ 0.2/ 6-7	6/ 6/ 0/ 6-6
9	16.9/ 17/ 2.1/ 12-24	26.0/ 26/ 2.7/ 20-30	25.2/ 25/ 1.9/ 20-29	21.0/ 21/1.6/ 17-24	8.3/ 8/ 2.8/ 6-22	28.1/ 28/ 1.9/ 24-32
10	6.3/ 6/ 1.5/ 6-15	25.6/ 25/ 1.6/ 22-29	22.8/ 24/ 3.2/ 8-27	19.9/ 20/ 2.7/ 10-28	6.4/ 6/ 2.5/ 6-21	27.6/ 28 4.1/ 6-32
11	6/ 6/ 0/ 6-6	24.9/ 25/ 1.5/ 22-29	23.8/ 24/ 2.3/ 20-31	21.1/ 21/ 1.7/ 18-26	21.8/ 22/ 1.8/ 17-25	23.7/ 24/ 2.1/ 20-29
12	16.3/ 17/ 2.4/ 6-18	25.4/ 26/ 1.7/ 20-28	8/ 8/ 1.4/ 6-10	11.6/ 12/ 2.1/ 6-21	21.5/ 22/ 2.4/ 15-25	6/ 6/ 0/ 6-6
13	18.5/ 19/ 2.0/ 10-21	27.1/ 27/ 1.6/ 24-30	6.1/ 6/ 0.6/ 6-9	19.8/ 20/ 1.0/ 18-22	6/ 6/ 0/ 6-6	6/ 6/ 0/ 6-6
14	6.5/ 6/ 1.5/ 6-13	22.3/ 22/ 2.3/ 18-28	6.2/ 6/ 0.6/ 6-8	21.8/ 21/ 3.0/ 8-26	6.5/ 6/ 0.7/ 6-8	18.3/ 19/ 3.0/ 6-22
15	6/ 6/ 0/ 6-6	26.9/ 27/ 1.5/ 24-30	8.5/ 8/ 2.6/ 6-21	19.2/ 20/ 2.4/ 11-22	22.8/ 24/ 4.5/ 6-28	6/ 6/ 0/ 6-6
16	6.0/ 6/ 0.2/ 6-7	28.8/ 29/ 2.7/ 23-33	22.8/ 23/ 2.3/ 17-27	8.1/ 8/ 1.2/ 6-10	23.3/ 23/ 1.9/ 19-27	6.0 /6/ 0.2/ 6-7
17	6/ 6/ 0/ 6-6	15.9/ 16/ 1.7/ 12-19	23.8/ 24/ 2.2/ 17-28	16.6/ 16/ 1.2/ 13-19	7.0/ 7/ 1.1/ 6-9	8.8/ 6/ 5.7/ 6-25
18	6/ 6/ 0/ 6-6	11.4/ 11/ 2.9/ 6-25	6/ 6/ 0/ 6-6	10.5/ 11/ 1.6/ 6-13	6.1/ 6/ 0.2/ 6-7	6/ 6/ 0/ 6-6
19	6.8/ 6/ 2.7/ 6-17	15.5/ 15/ 3.4/ 9-26	7.4/ 6/ 4.7/ 6-25	21.7/ 22/ 2.1/ 18-26	7.3/ 6/ 4.4/ 6-23	7.7/ 6/ 5.6/ 6-28
20	15.2/ 15/ 2.9/ 6-24	24.8/ 25/ 1.4/ 22-28	22.8/ 23/ 2.2/ 18-27	19.9/ 20/ 1.8/ 16-26	22.7/ 23/ 2.1/ 18-26	26.8/ 27/ 1.3/ 24-29

Supplementary Table 3. Performance of the disc diffusion assay from an inter- and intra-laboratory agreement study of seven veterinary laboratories where each laboratory assessed the susceptibility of 20 porcine *Escherichia coli* isolates to six antimicrobials on five occasions. EUCAST epidemiologic cut-off values (ECOFF) were used to categorise zone diameter values. Discordance occurred when an individual zone diameter disagreed with the median zone diameter for an isolate/ antimicrobial combination. ZD, zone diameter.

Antimicrobial	**Total no. of isolate/ antimicrobial combinations**	**No. of reported wild type replicates**	**No. of reported non wild-type replicates**	**No. of major errors[b]**	**No. of very major errors[b]**
Ampicillin*	700	213	487	13 (6.10%)	5 (1.03%)
Ceftiofur†	NA	NA	NA	NA	NA
Chloramphenicol*	700	348	358	13 (3.74%)	5 (1.40%)
Gentamicin†	700	399	301	26 (6.52%)	5 (1.67%)
Tetracycline*	700	211	489	4 (1.90%)	5 (1.02%)
Trimethoprim-Sulfamethoxazole*	NA	NA	NA	NA	NA
Total disagreement	2800	1171	1635	56 (4.78%)	20 (1.22%)

[b] Error rates are reported according to ISO 20776–2. Very major error rate is the number of false susceptible results divided by the number of isolates determined to be resistant (non-wildtype); Major error rate is the number of false resistant results divided by the number of isolates determined susceptible (wild-type).

NA, no published EUCAST epidemiologic cut-off value.

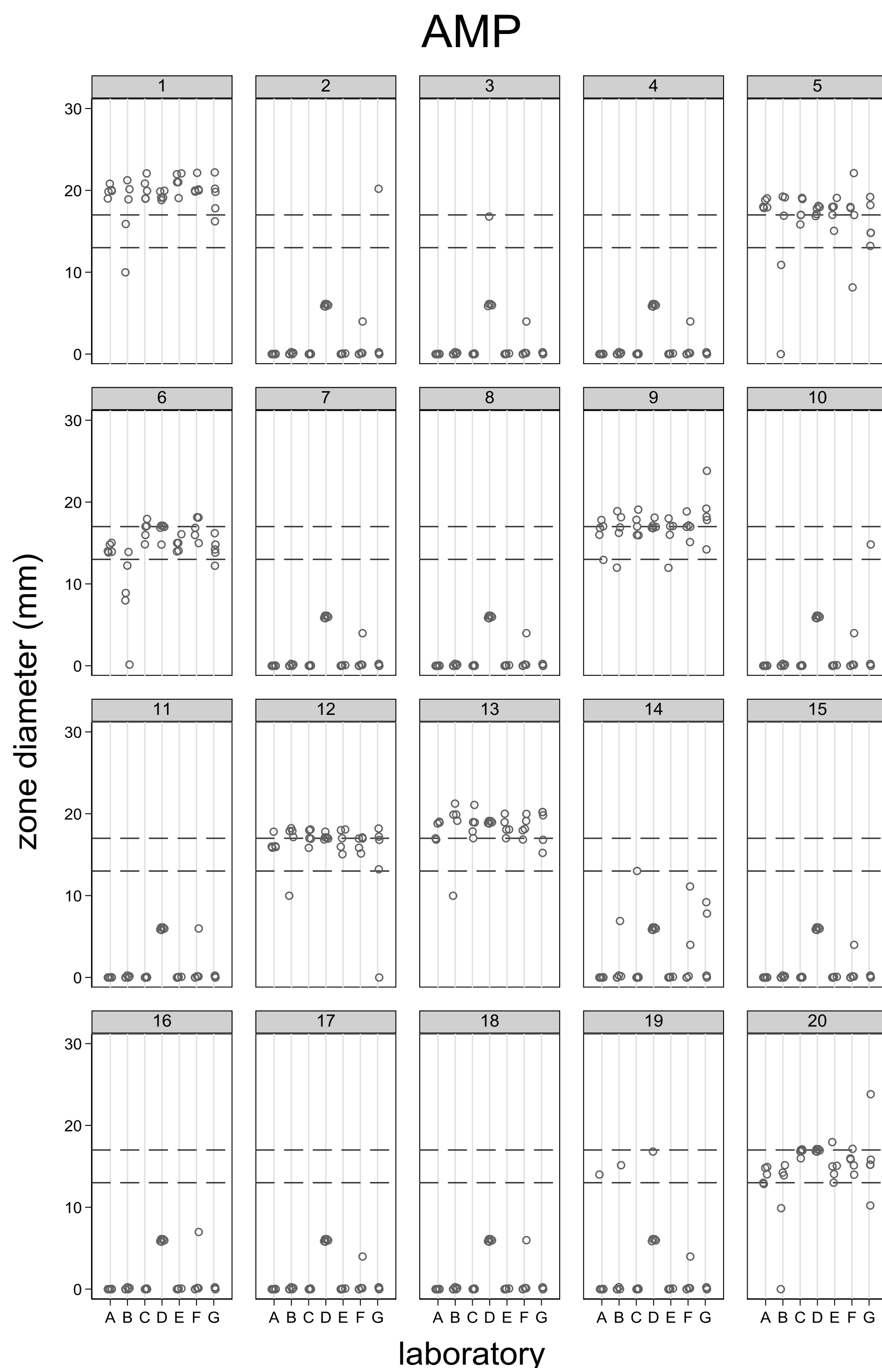
Supplementary Table 4. Within-subject values of coefficient of variation in the corrected zone diameter measurements (i.e. measurements <6mm were corrected to 6mm) for an inter- and intra-laboratory agreement study evaluating the disc diffusion assay within seven veterinary laboratories where each laboratory assessed the susceptibility of 20 porcine *Escherichia coli* isolates to six antimicrobials on five occasions.

	Coefficient of variance (%) with 95% CI[a]					
Isolate	**Ampicillin**	**Ceftiofur**	**Chloramphenicol**	**Gentamicin**	**Tetracycline**	**Trimethoprim-Sulfa**
1	11.2 (6.1, 18.5)	6.3 (5.1, 7.4)	5.2 (3.6, 7.2)	9.9 (5.1, 15.5)	5.3 (3.9, 7.3)	6.0 (4.5, 7.6)
2	37.0 (0, 48.5)	8.8 (6.6, 11.2)	25.7 (6.6, 11.2)	25.7 (14.0, 35.3)	7.7 (0, 11.3)	0.0 (0, 0)
3	30.0 (0, 39.1)	7.3 (5.8, 9.1)	48.8 (0, 74.1)	36.9 (15.1, 49.3)	0.0 (0, 0)	61.9 (0, 93.4)
4	0.0 (0, 0)	12.1 (4.5, 22.3)	8.3 (0, 17.6)	15.9 (13.0, 19.3)	0.0 (0, 0)	0.0 (0, 0)
5	18.6 (0.5, 28.3)	6.8 (4.7, 9.6)	6.6 (5.0, 8.4)	6.5 (5.5, 7.6)	2.8 (0, 3.9)	24.3 (18.8, 34.1)
6	18.3 (11.6, 26.7)	9.0 (5.1, 13.6)	16.5 (9.3, 27.2)	9.9 (6.9, 13.8)	15.4 (7.5, 27.1)	15.6 (6.6, 28.5)
7	0.0 (0, 0)	10.8 (6.3, 17.4)	14.2 (12.5, 16.1)	14.8 (10.9, 19.6)	10.1 (6.8, 12.4)	0.0 (0, 0)
8	0.0 (0, 0)	8.6 (6.2, 11.4)	14.7 (7.5, 25.7)	14.8 (11.0, 19.6)	3.9 (0, 5.3)	0.0 (0, 0)
9	12.6 (8.6, 17.4)	10.4 (6.0, 15.2)	7.4 (5.8, 9.6)	7.6 (6.0, 9.5)	33.0 (15.4, 49.6)	6.8 (5.4, 8.4)
10	24.3 (0, 32.5)	6.1 (5.0, 7.6)	14.0 (7.0, 24.6)	13.8 (8.3, 20.5)	39.4 (0, 65.4)	14.7 (46, 22.9)
11	0.0 (0, 0)	6.0 (4.3, 7.7)	9.7 (7.3, 12.5)	8.0 (6.1, 10.3)	8.2 (6.3, 10.6)	9.0 (7.0, 11.4)
12	14.7 (6.3, 25.9)	6.6 (4.8, 8.9)	17.9 (15.1, 20.9)	17.9 (7.7, 28.7)	11.1 (8.6, 14.2)	0.0 (0, 0)
13	10.6 (5.9, 16.9)	5.8 (4.6, 6.9)	9.0 (0, 13.8)	5.1 (4.1, 6.3)	0.0 (0, 0)	0.0 (0, 0)
14	23.4 (8.7, 33.1)	10.1 (7.8, 13.1)	9.2 (0, 12.1)	14.4 (8.4, 23.5)	11.5 (8.8, 13.5)	16.5 (6.5, 26.5)
15	0.0 (0, 0)	5.5 (4.4, 6.7)	30.1 (14.9, 44.8)	12.7 (7.1, 18.4)	19.8 (6.4, 31.1)	0.0 (0, 0)
16	2.8 (0, 3.9)	9.4 (7.0, 11.7)	10.0 (7.1, 12.9)	14.7 (11.7, 17.9)	8.11 (6.4, 9.8)	2.8 (0, 3.9)
17	0.0 (0, 0)	10.9 (9.0, 13.4)	9.2 (6.5, 12.3)	7.1 (5.0, 9.5)	15.4 (13.2, 17.6)	65.5 (58.3, 73.1)
18	0.0 (0, 0)	25.4 (11.1, 38.6)	0.0 (0, 0)	15.4 (10.1, 21.0)	3.9 (0, 5.3)	0.0 (0, 0)
19	40.0 (0, 50.1)	21.7 14.1, 28.8)	63.5 (34.5, 75.5)	9.6 (8.0, 11.4)	59.8 (37.0, 70.3)	73.0 (0, 82.4)
20	19.0 (11.4, 27.3)	5.8 (4.6, 7.3)	9.5 (7.5, 11.6)	9.3 (6.6, 12.3)	9.2 (7.7, 11.2)	4.7 (3.8, 5.8)
Median CV	11.6	8.8	9.9	11.3	10.2	5.8

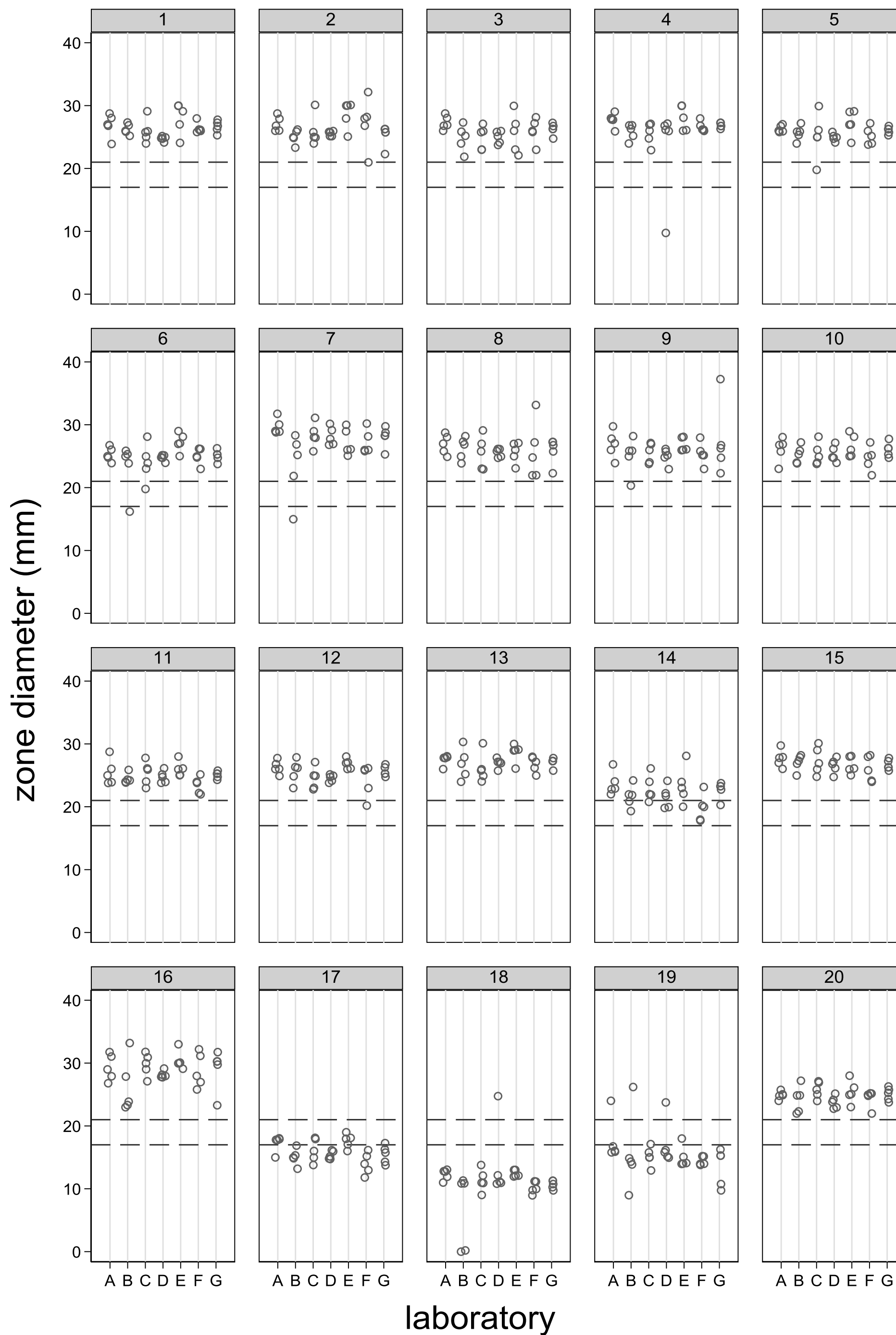
NA, not available.

[a] 95% confidence intervals derived Bias Corrected and Accelerated Interval using bootstrap estimation.

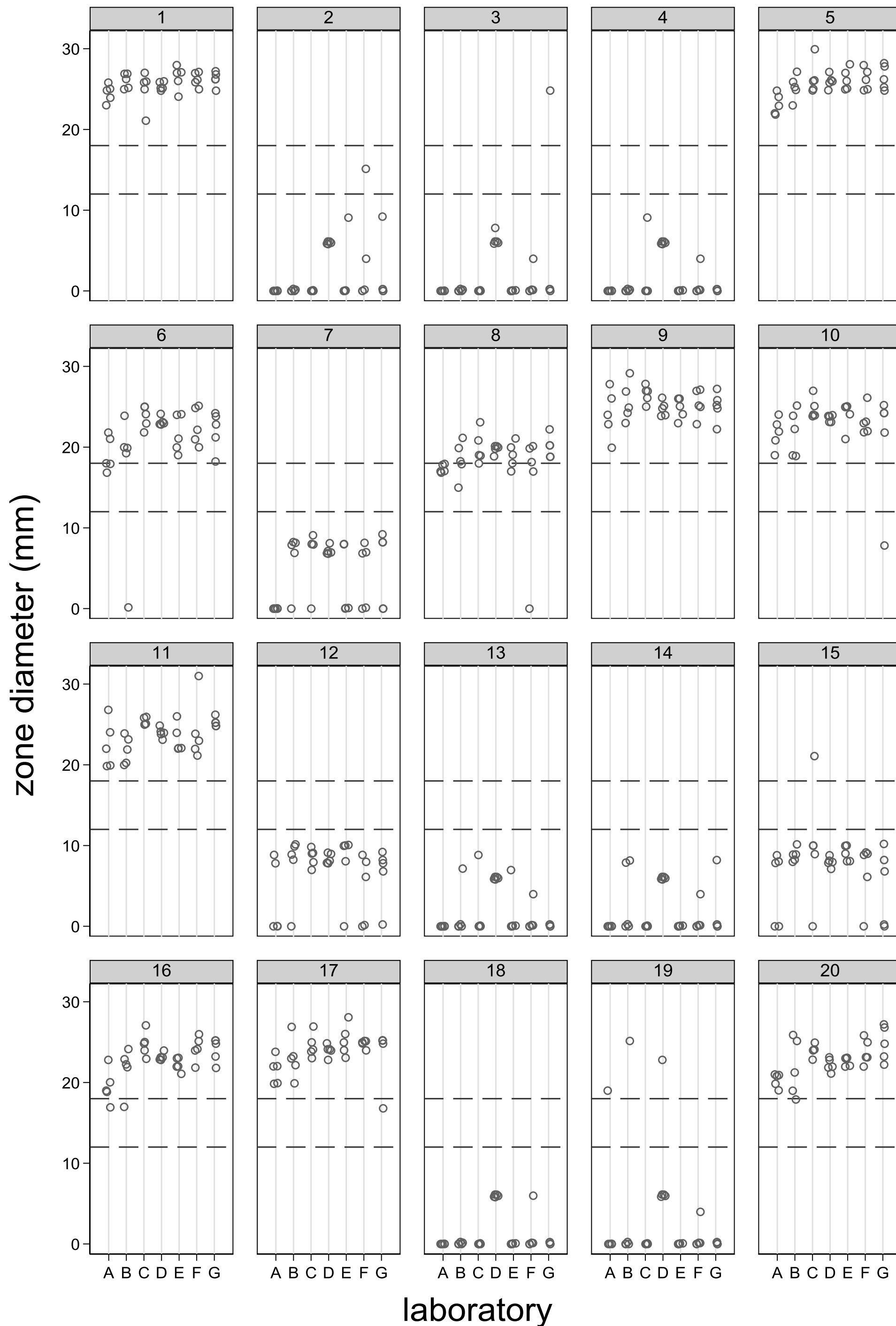
Supplementary Figures



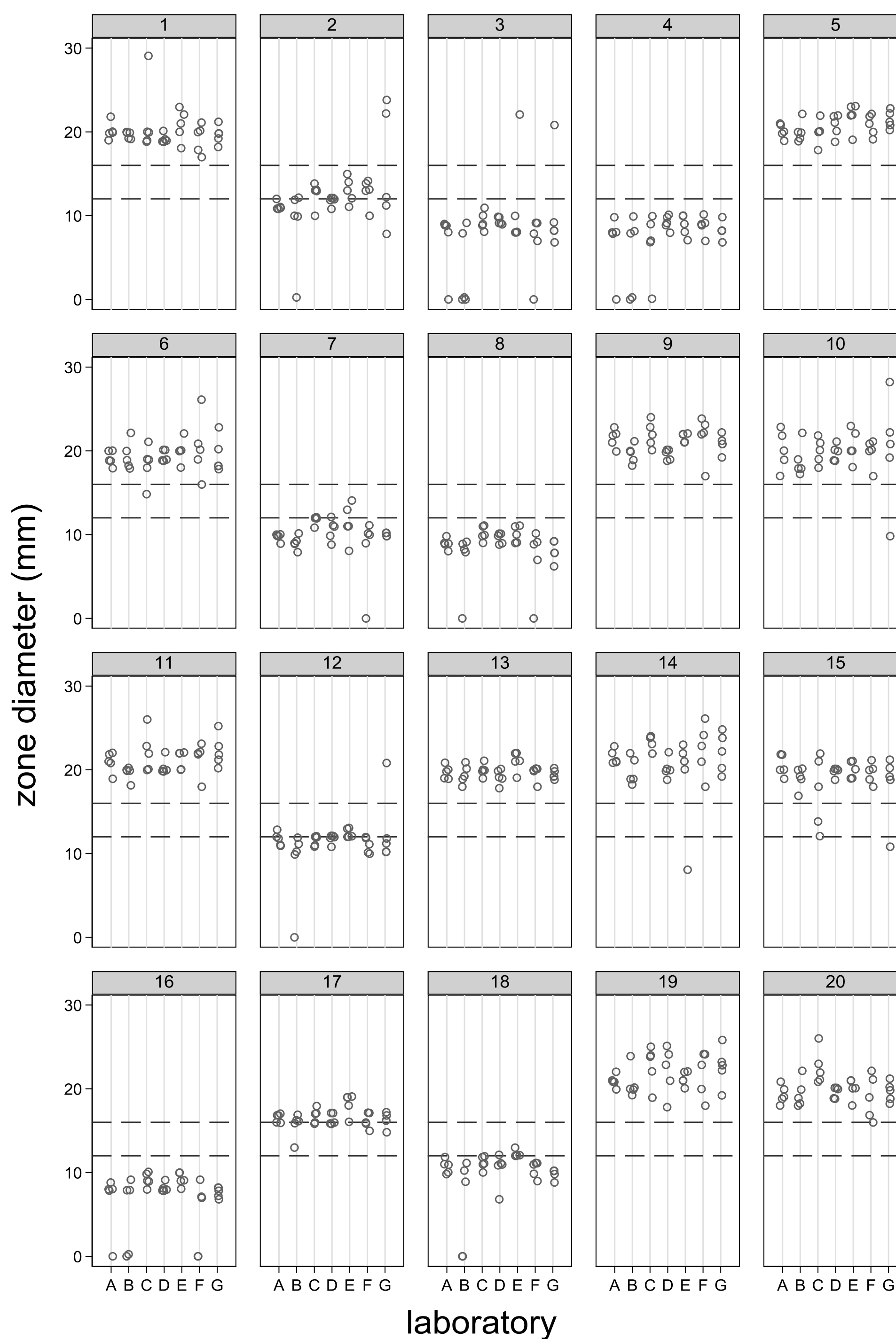
Supplementary Figure 1. Variation in repeated zone diameter measurements for ampicillin (AMP) for a panel of 20 porcine *Escherichia coli* isolates for an intra- and inter-laboratory agreement study assessing the disc diffusion assay. One plot is provided per isolate/ antimicrobial combination and represents five repeated measurements for each of 7 laboratories (A-G). Horizontal dashed lines represent Clinical and Laboratory Standards Institute (CLSI) susceptible and resistant clinical breakpoints relevant to each antimicrobial.



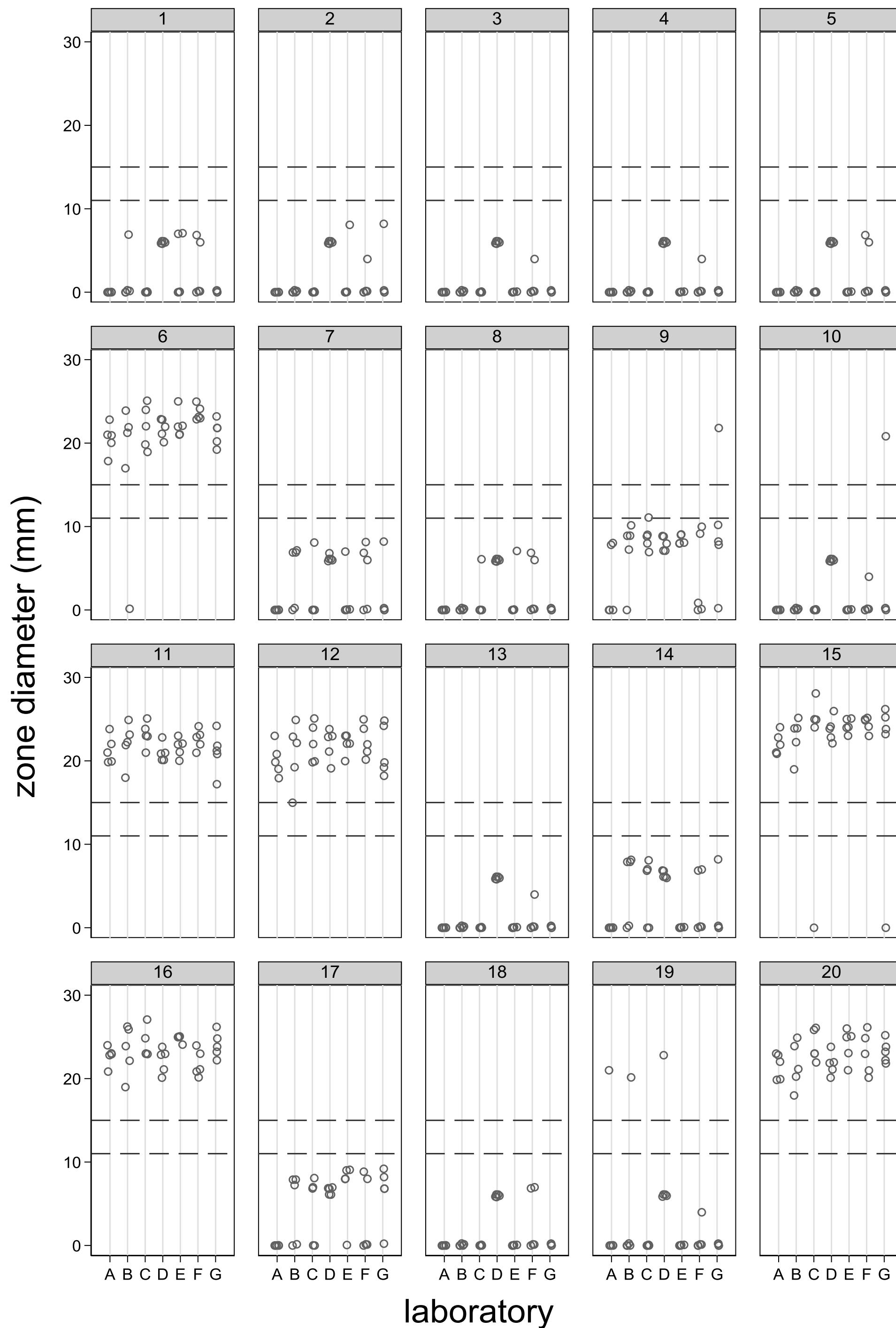
Supplementary Figure 2. Variation in repeated zone diameter measurements for ceftiofur (CFT) for a panel of 20 porcine *Escherichia coli* isolates for an intra- and inter-laboratory agreement study assessing the disc diffusion assay. One plot is provided per isolate/ antimicrobial combination and represents five repeated measurements for each of 7 laboratories (A-G). Horizontal dashed lines represent Clinical and Laboratory Standards Institute (CLSI) susceptible and resistant clinical breakpoints relevant to each antimicrobial.



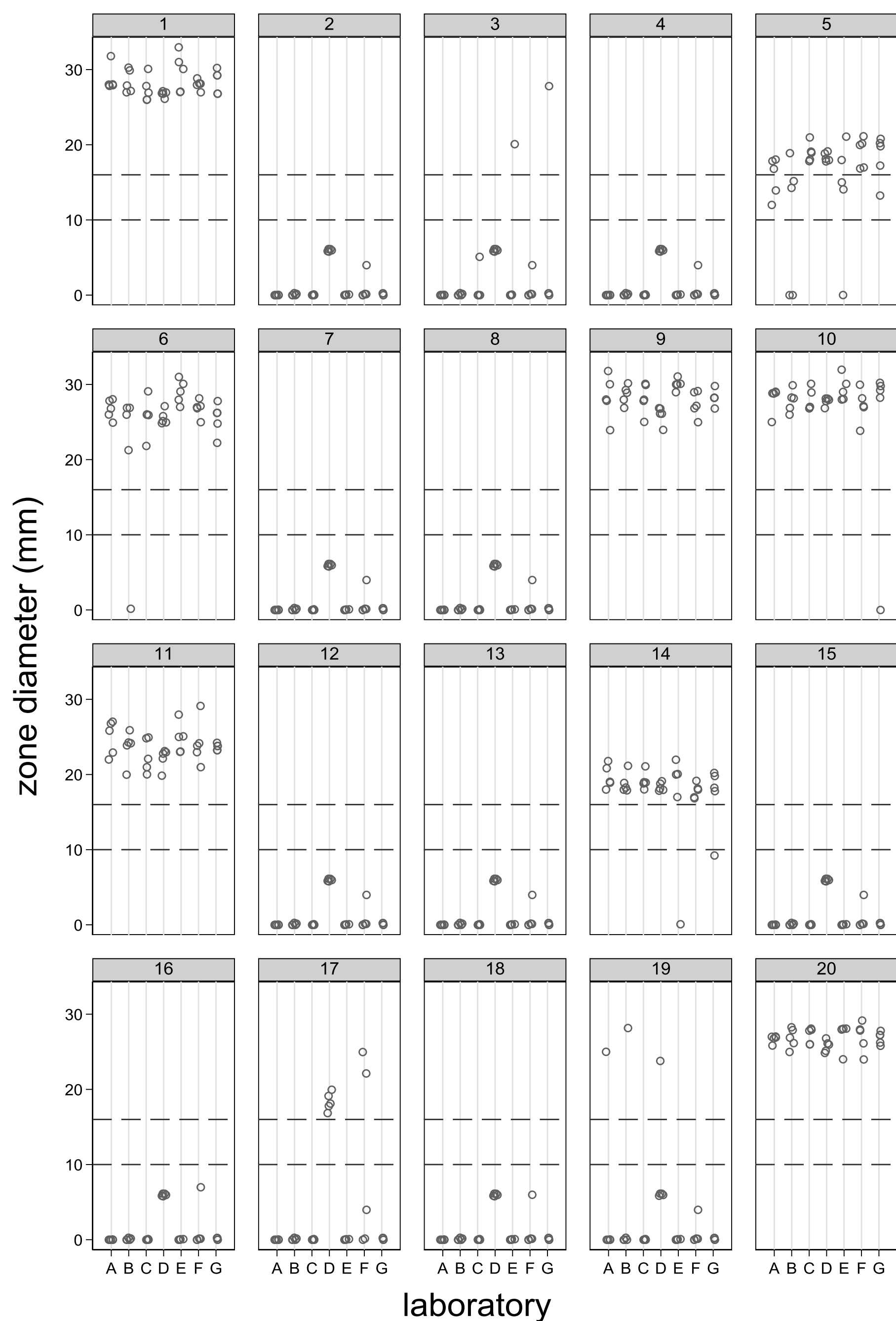
Supplementary Figure 3. Variation in repeated zone diameter measurements for chloramphenicol (CHL) for a panel of 20 porcine *Escherichia coli* isolates for an intra- and inter-laboratory agreement study assessing the disc diffusion assay**.** One plot is provided per isolate/ antimicrobial combination and represents five repeated measurements for each of 7 laboratories (A-G). Horizontal dashed lines represent Clinical and Laboratory Standards Institute (CLSI) susceptible and resistant clinical breakpoints relevant to each antimicrobial.



Supplementary Figure 4. Variation in repeated zone diameter measurements for gentamicin (GEN) for a panel of 20 porcine *Escherichia coli* isolates for an intra- and inter-laboratory agreement study assessing the disc diffusion assay. One plot is provided per isolate/ antimicrobial combination and represents five repeated measurements for each of 7 laboratories (A-G). Horizontal dashed lines represent Clinical and Laboratory Standards Institute (CLSI) susceptible and resistant clinical breakpoints relevant to each antimicrobial.



Supplementary Figure 5. Variation in repeated zone diameter measurements for tetracycline (TET) for a panel of 20 porcine *Escherichia coli* isolates for an intra- and inter-laboratory agreement study assessing the disc diffusion assay. One plot is provided per isolate/ antimicrobial combination and represents five repeated measurements for each of 7 laboratories (A-G). Horizontal dashed lines represent Clinical and Laboratory Standards Institute (CLSI) susceptible and resistant clinical breakpoints relevant to each antimicrobial.



Supplementary Figure 6. Variation in repeated zone diameter measurements for trimethoprim-sulfamethoxazole (SXT) for a panel of 20 porcine *Escherichia coli* isolates for an intra- and inter-laboratory agreement study assessing the disc diffusion assay. One plot is provided per isolate/ antimicrobial combination and represents five repeated measurements for each of 7 laboratories (A-G). Horizontal dashed lines represent Clinical and Laboratory Standards Institute (CLSI) susceptible and resistant clinical breakpoints relevant to each antimicrobial.

Appendix 4

Supplementary material from Chapter 5:

Antimicrobial use and stewardship practices on Australian beef feedlots

Beef Feedlot Questionnaire

Enter confidentiality code here ____________________

PART 1: GENERAL BACKGROUND INFORMATION

Select the feedlot size category that best describes your business

- ❍ < 3,000 animals
- ❍ 3,000-10,000 animals
- ❍ > 10,000 animals
- ❍ Unanswered

In the past 12 months, how many animals in total were sold from this feedlot?

- ❍ < 10,000
- ❍ 10,000 - 20,000
- ❍ 20,000-30,000
- ❍ 30,000 - 40,000
- ❍ >40,000
- ❍ Unanswered

In the past 12 months, what is the average time an animal will spend in the feedlot?

- ❍ <80 days
- ❍ 80-150 days
- ❍ > 150 days
- ❍ unanswered

In the past 12 months, what percentage of total animals in the feedlot were 'pulled' for treatment?

PART 2: ANITBIOTIC USE

Tylosin

Have you used tylosin by injection (trade names Bilosin, Tylan, Tylopharm) in the past 12 months?

- ❍ Yes
- ❍ No
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED ‘NO’, ‘DON’T KNOW’ OR ‘UNANSWERED’ SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of 'pulled' animals were given injectable tylosin in the past 12 months?

Nominate the purpose/s of using injectable tylosin (individual treatment, mass treatment response, timed or scheduled treatment) and the disease syndromes treated

	Individual	mass treatment	timed/ scheduled treatment
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

In-feed tylosin

Have you used in-feed tylosin (trade names Tylan, Tyleco) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of lots were given in-feed tylosin in the past 12 months?

____

Select the reason/s in-feed tylosin was used in the past 12 months (select multiple options)

- ❑ mass treatment in response to a disease outbreak (metaphylaxis)
- ❑ timed/ scheduled treatment of lots (prophylaxis)
- ❑ prevention
- ❑ growth promotion
- ❑ don't know
- ❑ unanswered

For the past 12 months, select the disease syndromes in-feed tylosin was for –

(1) mass treatment, and/or

(2) times/ scheduled treatment, and/or

(3) prevention

	mass treatment	timed/ scheduled treatment	prevention
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

Tilmicosin

Have you used tilmicosin by injection (trade names Micotil, Tilmax) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED ‘NO’, ‘DON’T KNOW’ OR ‘UNANSWERED’ SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of 'pulled' animals were given injectable tilmicosin in the past 12 months?

____

Nominate the purpose/s of using injectable tilmicosin (individual treatment, mass treatment response, timed or scheduled treatment) and the disease syndromes treated

	Individual	mass treatment	timed/ scheduled treatment
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

In-feed tilmicosin

Have you used in-feed tilmicosin (trade names Micotil, Tilmax) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED ‘NO’, ‘DON’T KNOW’ OR ‘UNANSWERED’ SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of lots were given in-feed tilmicosin in the past 12 months?

____

Select the reason/s in-feed tilmicosin was used in the past 12 months (select multiple options)

- ❑ mass treatment in response to a disease outbreak (metaphylaxis)
- ❑ timed/ scheduled treatment of lots (prophylaxis)
- ❑ prevention
- ❑ growth promotion
- ❑ don't know
- ❑ unanswered

For the past 12 months, select the disease syndromes in-feed tilmicosin was used for –

(1) mass treatment, and/or
(2) times/ scheduled treatment, and/or
(3) prevention

	mass treatment	timed/ scheduled treatment	prevention
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

Injectable erythromycin

Have you used erythromycin by injection (trade names Erymicin, Gallimycin) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of 'pulled' animals were given injectable erythromycin in the past 12 months?

____

Nominate the purpose/s of using injectable erythromycin (individual treatment, mass treatment response, timed or scheduled treatment) and the disease syndromes treated

	Individual	mass treatment	timed/ scheduled treatment
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

Tulathromycin

Have you used tulathromycin by injection (trade name Draxxin) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of 'pulled' animals were given injectable tulathromycin in the past 12 months?

____

Nominate the purpose/s of using injectable tulathromycin (individual treatment, mass treatment response, timed or scheduled treatment) and the disease syndromes treated

	Individual	mass treatment	timed/ scheduled treatment
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

Short-acting oxytetracycline

Have you used short-acting oxytetracycline by injection (trade names Alamycin, Engemycin, Terramycin 100, Tetravet 10) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of 'pulled' animals were given injectable short-acting oxytetracycline in the past 12 months?

____

Nominate the purpose/s of using injectable short-acting oxytetracycline (individual treatment, mass treatment response, timed or scheduled treatment) and the disease syndromes treated

	Individual	mass treatment	timed/ scheduled treatment
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

Long-acting oxytetracycline

Have you used long-acting oxytetracycline by injection (trade names Alamycin LA, Bicatop LA, Hexazol LA, Oxytet 200 LA, Terramycin/LA, Tetravet 200 LA) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of 'pulled' animals were given injectable long-acting oxytetracycline in the past 12 months?

____

Nominate the purpose/s of using injectable long-acting oxytetracycline (individual treatment, mass treatment response, timed or scheduled treatment) and the disease syndromes treated

	Individual	mass treatment	timed/ scheduled treatment
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

In-feed oxytetracycline or chlortetracycline

Have you used in-feed oxytetracycline or chlortetracycline (trade names CTC200, Oxy-Eco 100, Tetravet 980, Terramycin 200, Terramycin 880) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED ‘NO’, ‘DON’T KNOW’ OR ‘UNANSWERED’ SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of lots were given in-feed oxytetracycline or chlortetracycline in the past 12 months?

Select the reason/s in-feed oxytetracycline or chlortetracycline was used in the past 12 months (select multiple options)

- ❑ mass treatment in response to a disease outbreak (metaphylaxis)
- ❑ timed/ scheduled treatment of lots (prophylaxis)
- ❑ prevention
- ❑ growth promotion
- ❑ don't know
- ❑ unanswered

For the past 12 months, select the disease syndromes in-feed oxytetracycline or chlortetracycline was used for

(1) mass treatment and/or

(2) times/ scheduled treatment, and/or

(3) prevention

	mass treatment	timed/ scheduled treatment	prevention
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

Short-acting ceftiofur

Have you used short-acting ceftiofur by injection (trade names Calefur, Excenel, Norocef) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED ‘NO’, ‘DON’T KNOW’ OR ‘UNANSWERED’ SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of 'pulled' animals were given injectable short-acting ceftiofur in the past 12 months?

____

Nominate the purpose/s of using injectable short-acting ceftiofur (individual treatment, mass treatment response, timed or scheduled treatment) and the disease syndromes treated

	Individual	mass treatment	timed/ scheduled treatment
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

Long-acting ceftiofur

Have you used long-acting ceftiofur by injection (trade names Excede) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of 'pulled' animals were given injectable long-acting ceftiofur in the past 12 months?

Nominate the purpose/s of using injectable long-acting ceftiofur (individual treatment, mass treatment response, timed or scheduled treatment) and the disease syndromes treated

	Individual	mass treatment	timed/ scheduled treatment
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

Florfenicol

Have you used florfenicol by injection (trade names Nuflor) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of 'pulled' animals were given injectable florfenicol in the past 12 months?

Nominate the purpose/s of using injectable florfenicol (individual treatment, mass treatment response, timed or scheduled treatment) and the disease syndromes treated

	Individual	mass treatment	timed/ scheduled treatment
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

Short-acting penicillin

Have you used short-acting penicillin by injection (trade names Depocillin, Norocillin SA, Penethaject, Propercillin) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of 'pulled' animals were given injectable short-acting penicillin in the past 12 months?

____

Nominate the purpose/s of using injectable short-acting penicillin (individual treatment, mass treatment response, timed or scheduled treatment) and the disease syndromes treated

	Individual	mass treatment	timed/ scheduled treatment
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

Long-acting penicillin

Have you used long-acting penicillin by injection (trade names Benacillin, Norocillin LA, Ultrapen LA) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of 'pulled' animals were given injectable long-acting penicillin in the past 12 months?

____

Nominate the purpose/s of using long-acting penicillin (individual treatment, mass treatment response, timed or scheduled treatment) and the disease syndromes treated

	Individual	mass treatment	timed/ scheduled treatment
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

Amoxicillin

Have you used amoxicillin by injection (trade names Betamox, Bimoxyl, Bomox, Moxylan) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of 'pulled' animals were given injectable amoxicillin in the past 12 months?

____

Nominate the purpose/s of using injectable amoxicillin (individual treatment, mass treatment response, timed or scheduled treatment) and the disease syndromes treated

	Individual	mass treatment	timed/ scheduled treatment
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

Trimethoprim and/or sulphonamides

Have you used trimethoprim and/or sulphonamides by injection (trade names Amphoprim, SD333 Sulfadimidine, TMPS 240, Tribactral, Triprim, Trisoprim 480, Trivetrin) in the past 12 months?

❍ yes
❍ no
❍ don't know
❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED ‘NO’, ‘DON’T KNOW’ OR ‘UNANSWERED’ SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of 'pulled' animals were given injectable trimethoprim/ sulphonamides in the past 12 months?

____

Nominate the purpose/s of using injectable trimethoprim/ sulphonamides (individual treatment, mass treatment response, timed or scheduled treatment) and the disease syndromes treated

	Individual	mass treatment	timed/ scheduled treatment
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

In-feed trimethoprim and/or sulphonamides

Have you used in-feed trimethoprim and/or sulphonamides (trade names Sulphatrim, Sulprim, Trimidine) in the past 12 months?

❍ yes
❍ no
❍ don't know
❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of lots were given in-feed trimethoprim/ sulphonamides in the past 12 months?

____

Select the reason/s in-feed trimethoprim/ sulphonamides were used in the past 12 months (select multiple options)

- ❑ mass treatment in response to a disease outbreak (metaphylaxis)
- ❑ timed/ scheduled treatment of lots (prophylaxis)
- ❑ prevention
- ❑ growth promotion
- ❑ don't know
- ❑ unanswered

For the past 12 months, select the disease syndromes in-feed trimethoprim/ sulphonamides was used for –

(1) mass treatment, and/or
(2) times/ scheduled treatment, and/or
(3) prevention

	mass treatment	timed/ scheduled treatment	prevention
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

Neomycin

Have you used neomycin by injection (trade names Neomycin-penicillin, neomycin sulphate) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of 'pulled' animals were given injectable neomycin in the past 12 months?

____

Nominate the purpose/s of using injectable neomycin (individual treatment, mass treatment response, timed or scheduled treatment) and the disease syndromes treated

	Individual	mass treatment	timed/ scheduled treatment
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

Gentamicin

Have you used gentamicin by injection (trade names Gentam, Gentamax) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED ‘NO’, ‘DON’T KNOW’ OR ‘UNANSWERED’ SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of 'pulled' animals were given injectable gentamicin in the past 12 months?

____

Nominate the purpose/s of using injectable gentamicin (individual treatment, mass treatment response, timed or scheduled treatment) and the disease syndromes treated

	Individual	mass treatment	timed/ scheduled treatment
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

Enrofloxacin

Have you used enrofloxacin by injection (trade names Baytril, Enrotril) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED ‘NO’, ‘DON’T KNOW’ OR ‘UNANSWERED’ SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of 'pulled' animals were given injectable enrofloxacin in the past 12 months?

____

Nominate the purpose/s of using injectable enrofloxacin (individual treatment, mass treatment response, timed or scheduled treatment) and the disease syndromes treated

	Individual	mass treatment	timed/ scheduled treatment
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

In-feed virginiamycin

Have you used in-feed virginiamycin (trade name Eskalin) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of lots were given in-feed virginiamycin in the past 12 months?

____

Select the reason/s in-feed virginiamycin was used in the past 12 months (select multiple options)

- ❑ mass treatment in response to a disease outbreak (metaphylaxis)
- ❑ timed/ scheduled treatment of lots (prophylaxis)
- ❑ prevention
- ❑ growth promotion
- ❑ don't know
- ❑ unanswered

For the past 12 months, select the disease syndromes in-feed virginiamycin was used for –

(1) mass treatment, and/or

(2) times/ scheduled treatment, and/or

(3) prevention

	mass treatment	timed/ scheduled treatment	prevention
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

In-feed monensin

Have you used in-feed monensin (trade name Elancoban, Moneco, PhibroMonensin, Rumensin) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of lots were given in-feed monensin in the past 12 months?

____

Select the reason/s in-feed monensin was used in the past 12 months (select multiple options)

- ❑ mass treatment in response to a disease outbreak (metaphylaxis)
- ❑ timed/ scheduled treatment of lots (prophylaxis)
- ❑ prevention
- ❑ growth promotion
- ❑ don't know
- ❑ unanswered

For the past 12 months, select the disease syndromes in-feed monensin was used for –

(1) mass treatment, and/or
(2) times/ scheduled treatment, and/or
(3) prevention

	mass treatment	timed/ scheduled treatment	prevention
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

In-feed salinomycin

Have you used in-feed salinomycin (trade name Posistac, Saleco) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of lots were given in-feed salinomycin in the past 12 months?

Select the reason/s in-feed salinomycin was used in the past 12 months (select multiple options)

- ❑ mass treatment in response to a disease outbreak (metaphylaxis)
- ❑ timed/ scheduled treatment of lots (prophylaxis)
- ❑ prevention
- ❑ growth promotion
- ❑ don't know
- ❑ unanswered

For the past 12 months, select the disease syndromes in-feed salinomycin was used for –

(1) mass treatment, and/or
(2) times/ scheduled treatment, and/or
(3) prevention

	mass treatment	timed/ scheduled treatment	prevention
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

In-feed lasalocid

Have you used in-feed lasalocid (trade name Bovatec) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of lots were given in-feed lasalocid in the past 12 months?

____

Select the reason/s in-feed lasolacid was used in the past 12 months (select multiple options)

- ❑ mass treatment in response to a disease outbreak (metaphylaxis)
- ❑ timed/ scheduled treatment of lots (prophylaxis)
- ❑ prevention
- ❑ growth promotion
- ❑ don't know
- ❑ unanswered

For the past 12 months, select the disease syndromes in-feed lasolacid was used for –

(1) mass treatment, and/or

(2) times/ scheduled treatment, and/or

(3) prevention

	mass treatment	timed/ scheduled treatment	prevention
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

In-feed narasin

Have you used in-feed narasin (trade name Maxiban, Monteban) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of lots were given in-feed narasin in the past 12 months?

____

Select the reason/s in-feed narasin was used in the past 12 months (select multiple options)

- ❑ mass treatment in response to a disease outbreak (metaphylaxis)
- ❑ timed/ scheduled treatment of lots (prophylaxis)
- ❑ prevention
- ❑ growth promotion
- ❑ don't know
- ❑ unanswered

For the past 12 months, select the disease syndromes in-feed narasin was used for –
(1) mass treatment, and/or
(2) times/ scheduled treatment, and/or
(3) prevention

	mass treatment	timed/ scheduled treatment	prevention
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

In-feed flavophospholipol

Have you used in-feed flavophospholipol (trade name Flaveco) in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

IF ANSWERED YES PROCEED TO NEXT QUESTION. IF ANSWERED 'NO', 'DON'T KNOW' OR 'UNANSWERED' SKIP TO NEXT ANTIMICROBIAL AGENT

What percentage of lots were given in-feed flavophospolipol in the past 12 months?

____

Select the reason/s in-feed flavophospholipol was used in the past 12 months (select multiple options)

- ❑ mass treatment in response to a disease outbreak (metaphylaxis)
- ❑ timed/ scheduled treatment of lots (prophylaxis)
- ❑ prevention
- ❑ growth promotion
- ❑ don't know
- ❑ unanswered

For the past 12 months, select the disease syndromes in-feed flavophospholipol was used for –

(1) mass treatment, and/or

(2) times/ scheduled treatment, and/or

(3) prevention

	mass treatment	timed/ scheduled treatment	prevention
Respiratory (e.g. laryngitis, pneumonia)	❑	❑	❑
Gut (e.g. acidosis, blood scours, scours, rumen health)	❑	❑	❑
Musculoskeletal (lameness, footrot, swollen joints/ legs, foot abscess, lumpy jaw, wooden tongue)	❑	❑	❑
neurological (e.g. PEM/TEME, dropped ear, circling, pink eye)	❑	❑	❑
Urogenital (e.g. prolapsed prepuce, calving, waterbelly, castration)	❑	❑	❑
Other (e.g. tick fever, honker)	❑	❑	❑
Specify other:			

PART 3: VETERINARY TREATMENT CONTROLS

How often does a registered veterinarian visit the feedlot?

- ❍ never
- ❍ once a year
- ❍ twice a year
- ❍ four times a year
- ❍ monthly
- ❍ more than once a month
- ❍ unanswered

Does the feedlot have a protocol such as a 'documented processing protocol' for inducting new animals into the feedlot?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

Has a veterinarian issued the feedlot with a treatment protocol/ schedule or 'prescribed veterinary medicine and veterinary chemical list' in the past 12 months?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

Is the treatment protocol/ 'prescribed list' followed by feedlot staff? (select the most appropriate option)

- ❍ always (100% of the time)
- ❍ frequently (approx 80% of the time)
- ❍ often (approx 60% of the time)
- ❍ occasionally (approx 40% of the time)
- ❍ seldom (
- ❍ never
- ❍ unanswered

Are sick animals assessed for their response to treatments before they are returned to their 'home pen'?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

PART 4: SUPPLY AND USE OF VETERINARY CHEMICALS

Who supplies veterinary prescription drugs (e.g. S4 chemicals such as antimicrobial agents, anti-inflammatories) for use in the feedlot (can select multiple options)

- ❑ consulting vet
- ❑ other vet
- ❑ online
- ❑ don't know
- ❑ unanswered

Where do you buy animal health products such as drenches and pesticides (i.e. over-the-counter products) for use in the feedlot? (can select multiple options)

- ❑ vet
- ❑ online
- ❑ rural merchandise store
- ❑ other ____________________
- ❑ don't know
- ❑ unanswered

Who has access to veterinary prescription drugs at the feedlot? (can select multiple options)

- ❑ animal health crew/ stock handlers
- ❑ feeding crew/ maintenance crew
- ❑ management
- ❑ Other ____________________
- ❑ don't know
- ❑ unanswered

Who can access animal health products such as drenches and pesticides at the feedlot? (can select multiple options)

- ❑ animal health crew/ stock handlers
- ❑ feeding crew/ maintenance crew
- ❑ management
- ❑ Other ____________________
- ❑ don't know
- ❑ unanswered

Who can administer veterinary prescription drugs to animals at the feedlot? (can select multiple options)

- ❑ animal health crew/ stock handlers
- ❑ feeding crew/ maintenance crew
- ❑ management
- ❑ Other ____________________
- ❑ don't know
- ❑ unanswered

Who administers animal health products (drenches, pesticides) to animals at the feedlot? (can select multiple options)

- ❑ animal health crew/ stock handlers
- ❑ feeding crew/ maintenance crew
- ❑ management
- ❑ Other ____________________
- ❑ don't know
- ❑ unanswered

How do you identify animals that have been treated with a prescription drug (with an applicable withholding period)? (select one option)

- ❍ hospital tag only
- ❍ management computer software only
- ❍ hospital tag AND management computer software
- ❍ do not identify treated animals
- ❍ other ____________________
- ❍ don't know
- ❍ unanswered

What training is provided to staff who administer veterinary prescription drugs (e.g. antimicrobial agents, anti-inflammatories) to animals? (can select multiple options)

- ❑ staff trained by the feedlot veterinarian
- ❑ staff trained by the livestock supervisor
- ❑ off-site courses, seminars such as ChemCert
- ❑ other ____________________
- ❑ don't know
- ❑ unanswered

PART 5: STORAGE AND CHEMICAL STOCK CONTROL

Is the main storage area for veterinary prescription drugs locked at all times?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

Are veterinary prescription drugs and animal health products stored according to the label directions e.g. if the drug requires refrigeration is it always kept refrigerated?

- ❍ yes
- ❍ no
- ❍ don't know
- ❍ unanswered

How is veterinary chemical (i.e. prescription and over-the-counter) inventory managed for incoming/ outgoing chemicals? (select one option)

- ❍ computerised records
- ❍ manual entry - record book
- ❍ no records
- ❍ other ____________________
- ❍ don't know
- ❍ unanswered

How often are veterinary chemical stocks audited? (select one option)

- ❍ never
- ❍ once a year
- ❍ twice a year
- ❍ four times a year
- ❍ monthly
- ❍ more than monthly
- ❍ unanswered

How are out-of-date veterinary chemicals managed? (select one option)

- ❍ immediate disposal
- ❍ vet approved short extension of shelf life
- ❍ used until complete
- ❍ other ____________________
- ❍ don't know
- ❍ unanswered

Appendix 5

Antimicrobial agent Use Risk Matrix: weightings and justifications

Antimicrobial agent Use Risk Matrix – Beef Feedlots

	Behaviour Risk				
Antimicrobial agent risk ↓	**Optimal (0-1)**	**Adequate (1-2)**	**Suboptimal (3-4)**	**Inadequate (4-5)**	**Non-compliant (5)**
High risk (4-5)	High	High	Extreme	Extreme	Extreme
Medium risk (3-4)	Medium	Medium	High	High	High
Some risk (2-3)	Medium	Medium	Medium	Medium	High
Low risk (1-2)	Low	Low	Medium	Medium	Medium
Negligible risk (0-1)	Low	Low	Low	Low	Low

Key:

VL: very low antimicrobial agent use risk

L: low antimicrobial agent use risk

M: moderate antimicrobial agent use risk

H: high antimicrobial agent use risk

E: extreme antimicrobial agent use risk

Behaviour ratings	**Optimal**	**Adequate**	**Suboptimal**	**Inadequate**	**Non-compliant**
Vet issued treatment protocol	Yes	Yes	Yes	Yes or No	No
Tx protocol instructions followed	Always	Mostly	Mostly	Not always	No
Animal weighed prior to treatment	Always	Always	No/ Not always	No/ Not always	No
Correct AB use – dose, route, duration	Always	Always	Always/ Mostly	No	Unknown
Tx recorded and WHP observed	Always	Always	Always/ Mostly	No	No
Likely overall score	**0-1**	**1-2**	**3-4**	**4-5**	**5**

Antimicrobial agent Use ratings	**Negligible risk**	**Low risk**	**Some risk**	**Medium risk**	**High risk**
Antimicrobial agent index:					
Estimated proportion population administered drug in previous 12months	0%	<2%	2-10%	10-20%	>20%
Importance weighting – unrated, low, med, high imp drug	Nil	Low	Low, Med, +/- high	Low - high	Low - high
Route of administration – in-feed or injectable	Nil	Injectable, +/- unrated in-feed drugs	Injectable, unrated in-feed drugs, low imp in-feed drugs	Injectable, in-feed drugs	Injectable, in-feed drugs
Overall antimicrobial agent index:					
Sum of all individual antimicrobial agent indices (positive values)	0	<10	10-40	40-70	>70
Likely overall score	**0**	**1-2**	**2-3**	**3-4**	**4-5**

Antimicrobial agent Use Risk Matrix

Purpose:

- Categorise the risk of antimicrobial use practices at the individual feedlot level.
- A communication tool for industry stakeholders to understand feedlot level risk profiles based on antimicrobial use practices
- Can be used to assist antimicrobial stewardship adoption in beef feedlot sector
- The matrix and risk index have a flexible format so they can be changed to reflect the changing risk profile of any of the factors included in the index as more data comes to hand (e.g. importance weightings applied to drugs).

Determination of antimicrobial agent use behaviour ratings

Based on the approach to assessing quality of antimicrobial use presented in Antimicrobial Stewardship Guidelines for the Australian Cattle Feedlot Industry(Meat & Livestock Australia 2018).

APPROPRIATE	
Optimal	• Treatment protocol is in place and is always followed. • Bodyweight is measured. • Indication is recorded • Correct antimicrobial agent is used, dosage, route, duration and WHP is followed
Adequate	• Treatment protocol is in place but is not always followed. • Bodyweight is measured • Indication is recorded • Correct antimicrobial agent is used, dosage, route, duration and WHP is followed
INAPPROPRIATE	
Suboptimal	• Treatment protocol is in place and is followed. • Bodyweight is not measured. **AND/OR** • Indication is not recorded. • Correct antimicrobial agent is used, dosage, route, duration and WHP is followed
Inadequate	• Treatment protocol is in place but is not always followed. **AND/OR** • Indication is not recorded. **AND/OR** • Bodyweight is not measured. **AND/OR** • Correct antimicrobial agent is not used, dosage, route or duration may not be optimal. WHP is not followed.
UNKNOWN	
Non-Compliant	Treatment protocol is not documented. Bodyweight is not measured. Indication is not recorded. Treatments are not recorded

Determination of antimicrobial agent use ratings

The antimicrobial agent use ratings are a measure to quantify the amount of antimicrobial agents (by injection and in-feed) used by a beef feedlot. It is based on four rankings or weightings:

1. Importance ranking of antimicrobial agents included in survey
2. Use of antimicrobial agents in animals in past 12 months
3. Frequency of antimicrobial agent use (by drug) in past 12 months
4. Antimicrobial agent treatment by disease syndrome, based on risk to human health

The index is used to assess the level of antimicrobial agent use in beef feedlot herds and identify potential risk factors contributing to high antimicrobial agent use in feedlot conditions.

Calculation of antimicrobial agent use ratings:

- Individual Drug index = (proportion treated) x (drug) x (route of administration)
- Herd index = sum of all individual drug indices (range 0 to infinity)
- See Tables 1 to 4 for categorisation of risk

Weightings applied for antimicrobial agent use ratings:

Antimicrobial agent class importance weightings:

- Weightings are based on Australian Strategic and Technical Advisory Group (ASTAG) ratings (2018). The higher the ASTAG rating the higher the weighting (Tables 1 and 2):
  - 3 = high importance drugs (ceftiofur, enrofloxacin)
  - 2 = medium importance drugs (trim-sulpha)
  - 1 = low importance drugs (tetracyclines, penicillins etc)
  - 0 = unrated drugs (ionophores, glycophospholipids)

Route of administration weightings:

- Weighting is based on antimicrobial risk to microbiota. The higher the weighting, the higher the risk of resistance developing in the microbiota. In-feed antimicrobial agents have a higher risk than injectable drugs. In-feed antimicrobial agents are usually fed for a long time period and the dose is often dependent on voluntary intake of feed. In contrast, injectable drugs are more commonly a once-off treatment with a short (or long) duration of action:
  - 2 = in-feed antimicrobials
  - 1 = injectable antimicrobials

Table 1. Importance ranking of antimicrobial agents based Australian Strategic and Technical Advisory Group (ASTAG) rankings on risk of AMR to human health

Drug	Class	ASTAG Rating	AB use index rating*
Ceftiofur – SA, LA	3rd gen cephalosporin	High	3.0
Enrofloxacin	Fluoroquinolone	High	3.0
Virginiamycin	Streptogramin	High	3.0
Trim-sulfa	Sulphonamides, DHFR inhibitors	Medium	2.0
Gentamicin	Aminoglycoside	Medium	2.0
Neomycin	Aminoglycoside	Low	2.0
Tylosin, Tilmicosin, Erythromycin, Tulathromycin	Macrolide	Low	2.0
Florfenicol	Amphenicol	Low	2.0
Oxytetracycline – SA, LA	Tetracycline	Low	1.0
Penicillin – SA, LA, amoxicillin	Penicillin	Low	1.0
Monensin, salinomycin, lasalocid, narasin	Ionophore	unrated	0
Flavophospholipol	Glycophospholipid	unrated	0 (?)

* AB use index weighting assigned ASATG rankings and risk of resistance gene transfer from animal bacteria to humans (See Table 2).

Table 2: Justification applied to Antimicrobial agent Use index rating according to the risk of AMR to human health

AB use ranking	Category	Justification*
0	Low importance drug, no reported resistance	Not ranked by ASTAG, no reported mechanism of resistance in humans or animals
1	Low importance drug, resistance reported	Transfer of resistance – via human and animal pathways, direct human infection
2	Medium importance drug, resistance reported	Transfer of resistance – via human and animal pathways, direct human infection
3	High importance drug, resistance reported	Transfer of resistance – via human and animal pathways, direct human infection

* Rating is based on ASTAG rankings, and transfer pathways of resistance elements in humans and animals.

Table 3. Use of antimicrobial agents in feedlot cattle in past 12 months

Ranking	Definition
0	Not antimicrobial agent use
1	Injection antimicrobial agent use only
2	In-feed antimicrobial agent use only
3	Injection and in-feed antimicrobial agent use

Table 4. Proportion of feedlot cattle treated with an antimicrobial agent (by drug) in past 12 months

Ranking	Definition	
0	0%	No use
1	0-2%	Not much use
2	2.1-10%	Some use
3	10.1-20%	Very much use
4	> 20%	A great deal of use

References

Australian Strategic and Advisory Group on Antimicrobial Resistance (ASTAG) 2018, *Importance Ratings and Summary of Antibacterial Uses in Human and Animal Health in Australia, Version 1.0* Commonwealth of Australia, Canberra, Australia.

Meat & Livestock Australia 2018, *Antimicrobial Stewardship Guidelines for the Australian Cattle Feedlot Industry*, MLA, Sydney, viewed 22/05/2019 2019, <https://www.mla.com.au/globalassets/mla-corporate/research-and-development/program-areas/animal-health-welfare-and-biosecurity/mla_antimicrobial-stewardship-guidelines.pdf>.