A survey of mastitis pathogens including antimicrobial susceptibility in southeastern Australian dairy herds

R. Dyson,¹ N. Charman,² A. Hodge,² S. M. Rowe,³ and L. F. Taylor^{2*}

¹Dairy Focus, 181 Wharparilla Drive, Echuca, Victoria, 3564, Australia

²Zoetis Australia, 5 Rider Blvd, Rhodes, New South Wales, 2138, Australia

³Faculty of Science, Sydney School of Veterinary Science, The University of Sydney, Camden, New South Wales 2570, Australia

ABSTRACT

The objectives for this study were to (1) describe the pathogen profile in quarters from cows with clinical mastitis and in cows with subclinical mastitis in southeastern Australia; and (2) describe antimicrobial susceptibility among isolated pathogens. As a secondary objective, we aimed to compare antimicrobial resistance prevalence in pathogens isolated from clinical and subclinical mastitis samples. A convenience sample of dairy herds ($n = 65$) from 4 regions in southeastern Australia (Gippsland, Northern Victoria, Tasmania, Western Victoria) were invited to submit milk samples from cows with clinical and subclinical mastitis over a 14-mo period (January 2011 to March 2012). Farmers were instructed to collect aseptic quarter milk samples from the first 10 cases of clinical mastitis for each month of the study. In addition, farmers submitted composite milk samples from cows with subclinical mastitis at 1 or 2 sampling occasions during the study period. Aerobic culture and biochemical tests were used to identify isolates. Isolates were classified as susceptible, intermediate, or resistant to a panel of antimicrobial agents based on the zone of growth inhibition around antimicrobial-impregnated disks, with antimicrobial resistance (AMR) classified as nonsusceptibility by combining intermediate and resistant groups into a single category. Generalized linear mixed models were used to compare the prevalence of AMR between clinical and subclinical mastitis isolates. For clinical mastitis samples ($n = 3,044$), 472 samples (15.5%) were excluded for contamination. Of the remaining samples ($n = 2,572$), the most common results were *Streptococcus uberis* (39.2%), no growth (27.5%), *Staphylococcus aureus* (10.6%), *Escherichia coli* (8.4%), and *Streptococcus dysgalactiae* (6.4%). For subclinical mastitis samples ($n = 1,072$), 425 (39.6%)

were excluded due to contamination. Of the remaining samples ($n = 647$), the most common results were no growth (29.1%), *Staph. aureus* (29.1%), and *Strep. uberis* (21.6%). The prevalence of AMR among common isolates was low for the majority of antimicrobial agents. Exploratory analysis found that the probability of *Staph. aureus* demonstrating resistance to penicillin was 5.16 times higher (95% confidence interval: 1.68, 15.88) in subclinical isolates relative to clinical *Staph. aureus* isolates. A similar association was observed for amoxicillin with subclinical *Staph. aureus* isolates being 4.70 times (95% confidence interval: 1.49, 14.75) more likely to be resistant than clinical *Staph. aureus* isolates. We concluded that the most common bacteria causing clinical mastitis in dairy herds in Australia is likely to be *Strep. uberis*, whereas *Staph. aureus* is likely to be the most common cause of subclinical mastitis. Despite decades of antimicrobial use to control these organisms, AMR appears to be uncommon.

Key words: mastitis, antimicrobial resistance, *Streptococcus uberis*, *Staphylococcus aureus*

INTRODUCTION

Mastitis is consistently ranked as one of the most significant diseases affecting dairy cattle worldwide (Halasa et al., 2007; Rollin et al., 2015) due to its effects on health, welfare, and productivity. It is estimated that udder health issues, of which mastitis is a major component, cost the Australian dairy industry A\$150M (US\$110M) per year (Brightling et al., 2018), and each individual case of clinical mastitis costs A\$360 (US\$260; S. Bullen, Dairy Australia, Melbourne, Australia, personal communication).

Understanding the pathogen profile for mastitis is critical to management. For example, understanding the industry and farm-level pathogen profiles for clinical and subclinical mastitis can inform treatment protocols and identify interventions to prevent new cases through the strategic targeting of likely reservoirs of infection. There have been no large surveys of mastitis etiol-

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*Corresponding author: lee.f.taylor@zoetis.com

ogy in Australia, especially in southeastern Australia, where a large proportion of the Australian dairy herd is located. Small surveys have described the prevalence of clinical mastitis pathogens in dairy farming regions in Australia over the past 40 yr, including herds from Queensland (Daniel et al., 1982), the Gippsland region of Victoria (Watson et al., 1996; Gunn et al., 1999), and New South Wales (Shum et al., 2009). The findings from these studies indicate that the etiology of clinical mastitis has changed over time, with a shift from pathogens that commonly cause contagious mastitis (i.e., *Staphylococcus aureus* and *Streptococcus agalactiae*) toward pathogens that are commonly found in the environment of cows, namely *Streptococcus uberis*. This is consistent with global trends in bovine mastitis in developed countries (Ruegg, 2017); however, the mix of environmental pathogens identified in previous Australian studies has varied between studies (Gunn et al., 1999; Shum et al., 2009).

Given the reliance on antimicrobials for the treatment and control of mastitis and the growing concern over the risk of antimicrobial resistance (AMR) and its effect on public health, surveillance for AMR is a recommendation under the Office International des Epizooties (OIE) strategy for combating AMR (WHO-OIE, 2016). The Australian dairy industry has implemented an antimicrobial stewardship program based on these guidelines (Dairy Australia, 2021). Surveillance for AMR is an important component of this strategy. Only one of the aforementioned Australian surveys, from 1982, included assessment of the susceptibility of cultured pathogens to common antimicrobial agents used for the treatment and control of mastitis (Daniel et al., 1982). In this study, *Staph. aureus* accounted for 58% of isolates, and *Strep. agalactiae* was the next most common pathogen, which was isolated from 19% of samples. Also in this study, 45% of *Staph. aureus* isolates were classified as resistant to penicillin, but no *Strep. agalactiae* isolates were resistant to penicillin. A more recent Australian study found no evidence of methicillin resistance among 203 mastitis-associated *Staph. aureus* isolates (Worthing et al., 2018). This is reassuring because *Staph. aureus* remains a significant mastitis pathogen in Australian dairy herds.

A limitation of this Australian dairy-derived *Staph. aureus* study was that it did not investigate other mastitis pathogens or other antimicrobial agents. Therefore, baseline studies of AMR prevalence among a broad spectrum of clinical and subclinical mastitis pathogens in Australian herds are required for ongoing monitoring of AMR among mastitis pathogens. In addition, describing pathogen profiles at the herd and industry levels for clinical and subclinical mastitis will provide

an insight into likely risk factors and inform current and future treatment protocols. For example, describing the proportion of clinical mastitis cases associated with gram-positive pathogens could be used to identify the cost-effectiveness of culture-guided, selective therapy for clinical mastitis under Australian conditions (Down et al., 2017; McDougall et al., 2018).

The objectives for this study were to (1) describe pathogen profiles in quarters from cows with clinical mastitis and in cows with subclinical mastitis in dairy farming regions of southeastern Australia; and (2) describe antimicrobial susceptibility among isolated pathogens. As a secondary objective, we aimed to compare AMR prevalence in pathogens isolated from clinical and subclinical mastitis samples.

MATERIALS AND METHODS

The Strengthening the Reporting of Observational Studies in Epidemiology – Veterinary Extension (STROBE-Vet) statement guidelines were followed in the reporting of this study (Sargeant et al., 2016). A cross-sectional study of mastitis etiology in southeastern Australian dairy cows was conducted between January 2011 and March 2012. This study was conducted under animal ethics approval authorized by the CSL Limited Pfizer Australia Animal Ethics Committee on October 11, 2010 (approval number 8,540). No sample size calculations were performed before the study.

Recruitment of Herds

A convenience sample of 65 herds from 3 dairy farming regions in Victoria (Western Victoria, Northern Victoria, and Gippsland) and Tasmania was enrolled with the assistance of 13 participating veterinary clinics. We chose to enroll herds from these regions as the Australian dairy industry is clustered in these areas. At the time that fieldwork commenced (2011), there were 6,770 dairy herds in Australia, with 4,556 (67.3%) located in Victoria and 444 (6.6%) in Tasmania, meaning this survey was conducted in regions that contained 73.9% of Australia's dairy herd. The average herd size of Victorian and Tasmanian dairy herds in 2011 was 232 and 328 cows, respectively. In 2011, the average bulk milk SCC in Australian dairy herds was 232,000 cells/mL, based on records from 5,976 herds. Of these, 61.0% of herds were $\leq 250,000$ cells/mL, 35.4% were $> 250,000$ cells/mL but $\leq 400,000$ cells/mL, and 3.5% were $> 400,000$ cells/mL (Taylor, 2021).

Participating veterinary clinics each selected 4 to 6 herds based on the following inclusion criteria: milking herd size > 250 cows, previous year's bulk milk SCC

averaging between 100,000 and 500,000 cells/mL, using electronic herd records, located within 50 km of the participating clinic, capable of delivering chilled milk samples to the participating clinic within 48 h of collection, and undertaking regular testing (>4 tests per lactation) of individual cow SCC with a herd-testing organization. On admission to the study, herd managers were asked to complete a questionnaire with questions about farm size, their production system, ration fed, dry cow antibiotic and internal teat sealant use at dry-off, and number of cows culled due to mastitis the previous year.

Recruitment of Samples

Clinical Mastitis. Farmers were instructed to collect milk samples from the first 10 eligible cases of clinical mastitis during each month of the study ($n = 13$). Clinical mastitis was defined as visibly abnormal milk after 3 strips of foremilk (clots, discoloration, watery), inflammatory signs in affected glands (swelling, heat, pain, redness), or both. Cows with clinical mastitis were eligible for inclusion if they met the following criteria: currently lactating, not treated for mastitis in the previous 28 d, not currently receiving treatment with antimicrobial agents or within the withholding period for antimicrobial treatments, and otherwise in good general health. Cows with multiple affected quarters were sampled in one quarter, which was decided by the farmer. Individual cows were eligible to be sampled in multiple months of the study.

Farm workers were instructed to collect milk samples using the following routine. With clean disposable gloves, farmers scrubbed teat ends with 70% isopropyl alcohol-soaked wipes until teat ends were clean (minimum 10 s), discarded 2 squirts of foremilk, and sampled approximately 2 to 4 mL of milk into sterile vials. Gloves were washed or replaced between cows. Samples were immediately chilled at 2 to 8°C and delivered to the local clinic within 48 h.

Subclinical Mastitis. Forty-seven participating farms also submitted milk samples from cows with subclinical mastitis at 1 or 2 sampling occasions during the 13-mo study period. Immediately after a herd test, data were downloaded by the study monitors. Infection was defined by a SCC above 250,000 as per Australian industry guidelines (Brightling et al., 2018). Cows were identified for sampling by study monitors from those with a range of SCC above the threshold (not just the highest), with a mix of “new” infections (as defined by a low previous SCC during lactation) and “old” infections (SCC had been elevated at prior herd tests conducted during current lactation), from a range of cow ages and stages of lactation, with the exception of cows in very

early lactation. Lists of suitable cows were sent to the farm and as many nominated cows were sampled as possible. Sampling method was the same as for clinical mastitis cows, except that composite samples were collected (i.e., milk from all functional quarters commingled into a single sample).

Bacteriology

Aerobic Culture. After arrival at the local veterinary clinic, milk samples were shipped immediately to an accredited (National Association of Testing Authorities, Australia) commercial veterinary diagnostic laboratory (Gribbles Veterinary Pathology, Clayton, Victoria, Australia). Samples were inoculated onto Columbia agar with 5% sheep blood with 1 loop-full (approximately 10 μ L) of sample using disposable plastic loops and then incubated in aerobic conditions at $37 \pm 2^\circ\text{C}$ for 48 h. Isolates were identified using growth characteristics on blood agar, microscopic appearance after staining (Gram and Giemsa), and biochemical testing. We identified *Staph. aureus* using catalase, coagulase (latex agglutination), and DNase activity. Gram-positive, catalase-negative species were identified using hemolytic patterns and Lancefield group testing, with group B isolates classified as *Strep. agalactiae*, group C isolates classified as *Strep. dysgalactiae*, and nongrouping isolates classified as *Strep. uberis*. Isolates classified as group B that had an atypical appearance (i.e., colonies were not hemolytic) were subject to further testing using the Christie–Atkins–Munch–Petersen (CAMP) test and *Streptococcus* API (bioMérieux Australia Pty Ltd.). Gram-negative isolates were identified using oxidase, indole, and Microbact gram-negative test kits (ThermoFisher). *Corynebacterium bovis* and *Listeria monocytogenes* were identified using API (bioMérieux Australia Pty Ltd.) along with colony morphology and first principles identification. *Trueperella pyogenes* were identified using Loeffler’s slope. No specialized culture medium and incubator settings were used for isolation of *Mycoplasma* spp. Mixed growths (i.e., 2 or more isolates from a single sample) were usually classified as contaminated. However, in situations when more than 1 mastitis pathogen was present in heavy growth in a milk sample, it was identified and reported but managed as a separate sample from the same cow. Consequently, only 1 mastitis pathogen was recorded for each sample.

Antimicrobial Susceptibility. Antimicrobial susceptibility was determined on each isolate using disk diffusion, according to methods described in the Clinical and Laboratory Standards Institute (CLSI) performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals—third edition (Watts et al., 2008). Isolates were

transferred after 18 to 24 h of growth onto Mueller-Hinton agar using a sterile swab and a 0.5 McFarland turbidity reference standard suspension. Antimicrobial-impregnated paper disks were applied with a dispenser. Susceptibility was evaluated for amoxicillin-clavulanic acid (20 and 10 µg, respectively), amoxicillin (20 µg), cefuroxime (30 µg), cloxacillin (5 µg), neomycin (30 µg), penicillin-novobiocin (10 and 30 µg, respectively), penicillin (10 µg), streptomycin (10 µg), tetracycline (30 µg), and trimethoprim-sulfamethoxazole (1.25 and 23.75 µg, respectively). These antimicrobial agents were chosen to match the antimicrobials used to treat and control mastitis in Australia (Taylor, 2021; Supplemental Table S1; <https://osf.io/dfq7p>). Different antimicrobial panels were used for pathogen groups according to testing protocols at the laboratory. *Staphylococcus aureus* isolates had susceptibility determined for amoxicillin, cefuroxime, cloxacillin, erythromycin, neomycin, penicillin, penicillin-novobiocin, tetracycline, and trimethoprim-sulfamethoxazole. *Streptococcus* spp. isolates had susceptibility determined for amoxicillin, cefuroxime, cloxacillin, erythromycin, penicillin, penicillin-novobiocin, tetracycline, and trimethoprim-sulfamethoxazole. Isolates belonging to the family *Enterobacteriaceae* had susceptibility determined for amoxicillin (excluding *Escherichia coli* and *Serratia* spp.), amoxicillin-clavulanate (excluding *Serratia* spp.), cefuroxime (excluding *Serratia* spp.), neomycin, streptomycin, tetracycline, and trimethoprim-sulfamethoxazole. *Pasteurella* spp. isolates had susceptibility determined for amoxicillin, amoxicillin-clavulanate, cefuroxime, penicillin, tetracycline, and trimethoprim-sulfamethoxazole. Zones of inhibition were visually measured using a ruler, and isolates were initially classified as susceptible, intermediate, or resistant based on CLSI recommendations. Quality control evaluation of zone inhibition diameters against reference isolates was undertaken weekly according to CLSI protocols. Resistance was classified as nonsusceptibility by combining intermediate and resistant groups into a single category (Nobrega et al., 2018b). Breakpoints for classification as susceptible for each bacterial isolate and antimicrobial combination are provided in Supplemental Table S2 (<https://osf.io/dfq7p>; Taylor, 2021). Methicillin susceptibility testing was not performed for any isolates, which prevented us from identifying the presence or absence of methicillin resistance. Antimicrobial susceptibility was not assayed for CNS.

Statistical Analysis

Statistical analyses were conducted using SAS for Windows version 9.4 (SAS Institute Inc.). The number

and percentage of each pathogen identified was summarized for clinical and subclinical isolates overall and stratified by region. Pathogen profiles based on percentages were also calculated at the herd level for herds that had ≥ 20 valid (i.e., noncontaminated) clinical samples. Prevalence and exact 95% confidence intervals (Clopper-Pearson confidence limits) for the AMR were presented for *E. coli*, *Serratia* spp., *Staph. aureus*, *Strep. dysgalactiae*, and *Strep. uberis* for clinical and subclinical isolates and for *Klebsiella* spp. and *Pasteurella* spp. for clinical isolates only. Trends in plots were explored visually.

As an exploratory analysis, the risk of AMR was compared between clinical and subclinical isolates for pathogen-antimicrobial combinations. This was only conducted for pathogen-antimicrobial combinations that had enough data to avoid issues with positivity [i.e., at least 20 clinical and 20 subclinical isolates and AMR prevalence of >0.01 (1%) in clinical or subclinical groups]. Consequently, the pathogen-antimicrobial combinations that were eligible for this exploratory analysis were as follows: *Staph. aureus* – penicillin (model 1), *Staph. aureus* – amoxicillin (model 2), *Staph. aureus* – erythromycin (model 3), *Strep. dysgalactiae* – erythromycin (model 4), *Strep. dysgalactiae* – tetracycline (model 5), *Strep. uberis* – erythromycin (model 6), and *Strep. uberis* – tetracycline (model 7). No adjustments were made for multiple comparisons.

In each case, the binary outcome variable denoting resistance was analyzed with a generalized linear mixed model with a Poisson distribution and log link. Poisson models were used to achieve estimates of risks and relative risks, rather than odds and odds ratios (Ospina et al., 2012). The model terms included the fixed effect of mastitis type (clinical or subclinical) and the random effect of farm. A random effect for cow was not included in final models because it was very uncommon to have multiple isolates per cow for each antibiotic-pathogen combination. No adjustments were made to address temporal clustering. Estimated risks have been presented for each mastitis type, along with the relative risk (with 95% CI) of a resistant result for subclinical samples compared with clinical samples.

RESULTS

Description of Enrolled Herds

The response rate to individual questions within the herd-demographics questionnaire ranged from 52 (80.0%) to 65 (100.0%). Average peak herd size (the maximum number of milking cows in the herd during the study period) was 464 cows (range 210–1,000; 64/65

Table 1. Pathogen profile for clinical mastitis samples from 65 southeastern Australian dairy farms collected between January 2011 and March 2012, stratified by region

Item	Gippsland (Victoria)		Northern Victoria		Tasmania		Western Victoria		All samples	
	n	%	n	%	n	%	n	%	n	%
Quarters submitted	911		912		132		1,089		3,044	
Contaminated	157	17.2	130	14.3	14	10.6	171	15.7	472	15.5
Quarters with valid results	754	100.0	782	100.0	118	100.0	918	100.0	2,572	100.0
No growth	186	24.7	240	30.7	44	37.3	237	25.8	707	27.5
Growth	568	75.3	542	69.3	74	62.7	681	74.2	1,865	72.5
Gram-positive										
CNS	1	0.1	6	0.8	1	0.8	6	0.7	14	0.5
<i>Corynebacterium bovis</i>	13	1.7	15	1.9	1	0.8	19	2.1	48	1.9
<i>Listeria monocytogenes</i>	1	0.1	1	0.1	0	0.0	0	0.0	2	0.1
<i>Nocardia</i> spp.	10	1.3	5	0.6	0	0.0	22	2.4	37	1.4
<i>Staphylococcus aureus</i>	115	15.3	41	5.2	16	13.6	100	10.9	272	10.6
<i>Streptococcus agalactiae</i>	3	0.4	0	0.0	0	0.0	1	0.1	4	0.2
<i>Streptococcus dysgalactiae</i>	77	10.2	26	3.3	7	5.9	55	6.0	165	6.4
<i>Streptococcus uberis</i>	251	33.3	315	40.3	40	33.9	402	43.8	1,008	39.2
<i>Trueperella pyogenes</i>	3	0.4	6	0.8	1	0.8	5	0.5	15	0.6
Gram-negative										
<i>Escherichia coli</i>	66	8.8	88	11.3	6	5.1	56	6.1	216	8.4
<i>Klebsiella</i> spp.	9	1.2	8	1.0	1	0.8	2	0.2	20	0.8
<i>Pasteurella</i> spp.	3	0.4	6	0.8	1	0.8	6	0.7	16	0.6
<i>Pseudomonas aeruginosa</i>	1	0.1	2	0.3	0	0.0	0	0.0	3	0.1
<i>Salmonella</i> spp.	0	0.0	2	0.3	0	0.0	1	0.1	3	0.1
<i>Serratia</i> spp.	9	1.2	7	0.9	0	0.0	3	0.3	19	0.7
Other bacteria	4	0.5	4	0.5	0	0.0	1	0.1	9	0.3
<i>Candida</i> spp. or yeasts	2	0.3	10	1.3	0	0.0	2	0.2	14	0.5

herds responded). Of the 52 farms reporting their calving pattern, 31 (59.6%) were classified as split calving (<3 calving groups/year), 16 (30.8%) were seasonal (the whole herd calved over a 2–3 mo period at the same time each year), 4 (7.7%) were year-round calving, and 1 (1.9%) was batch calving (>3 calving groups per year). Herds reported using rotary (54.7%) and herringbone (45.3%, cows stand at various angles up to 90 degrees to the pit) parlors (64/65 herd response). The most common feeding system was a combination of grazed pasture and grain-based concentrates fed in the milking parlor (64.1%), followed by partial mixed ration (26.6%), grazing-only (7.8%), and TMR (1.6%). Of the 60 farmers who reported the active agent in their teat sprays, all used an iodophore-based teat spray for postmilking teat disinfection. Of the 61 farmers reporting dry-off practices in the previous season, 85.2% and 14.8% practiced whole-herd (blanket) and part-herd (selective) dry cow therapy, respectively. Of 62 farmers who described the antimicrobial dry cow product used in the previous season, 75.8% used a cloxacillin-only product, 9.7% used both cloxacillin or cephalonium, 12.9% used cephalonium only, and 1.6% used either an ampicillin-cloxacillin combination product or cephalonium. Of 58 farms that reported on the use of an internal teat sealant on their farm, 56.9%

used an internal teat sealant as part of the drying off strategy.

Clinical Mastitis

A total of 3,073 quarter samples were submitted from cases of clinical mastitis from 65 different farms, but 29 records were excluded from all subsequent summaries as duplicate records (same date, animal and pathogen), leaving 3,044 unique samples as summarized in Table 1.

Description of Enrolled Cows and Cases. The 3,044 clinical samples came from 2,651 different cows as follows: 2,337 cows with a single sample, 69 cows with 2 to 3 different samples at 1 time point, and 245 cows with 1 or more samples at 2 to 5 different time points.

Clinical mastitis samples were submitted consistently throughout the first 12 mo of the survey at an average of 238 samples/month (range 140–378). Sample submissions were lower in the last 3 mo of the survey, averaging 65 samples/month. The average number of submissions per farm was 46.8 (range 5–197). Of 3,044 samples received from cows with clinical mastitis, 1,860 (61.1%) were Holstein, 170 (5.6%) were Jersey, 329 (10.8%) were various crosses of Holstein and Jersey, 61 (2.0%) were Aussie Reds (an Australian composite red dairy breed), and the remainder were a mix of purebred

Brown Swiss, Illawarra (an Australian red dairy breed), Ayrshire, or various crosses of these breeds. Breed was unknown for 185 samples (6.1%).

Based on 2,825 clinical mastitis samples submitted where the parity was recorded, 15.0% of cows were from first, 16.3% from second, 20.0% from third, 17.0% from fourth, 13.8% from fifth, 8.4% from sixth, and 9.5% from greater than seventh parity. Of 2,709 samples where stage of lactation was recorded, the number (%) of samples submitted from each stage of lactation were 0 to 1 mo (629, 23.2%), 2 to 6 mo (1,268, 46.8%), and ≥ 7 mo (812, 30.0%). The lactation stage profiles for each region were similar, except for Tasmania, which included a higher proportion of samples submitted from cows at 0 to 1 mo postcalving (53/99, 53.5%).

Pathogen Profile. Of the 3,044 clinical mastitis samples, 472 (15.5%) were excluded from analysis due to contamination. Of the remaining 2,572 samples with valid results, 707 (27.5%) did not identify a pathogen using standard bacteriological methods (Table 1). *Streptococcus uberis* was the most common pathogen isolated from clinical mastitis samples, with 39.2% of valid results attributable to this bacterium. The next most common pathogens recorded were *Staph. aureus*, *E. coli*, and *Strep. dysgalactiae*, with 10.6%, 8.4% and 6.4% recorded respectively. *Streptococcus agalactiae* was only cultured from 4 clinical mastitis samples (0.2%).

Prevalence of common pathogens (*Strep. uberis*, *Staph. aureus*, *E. coli*, *Strep. dysgalactiae*, *Corynebacterium bovis*) stratified by stage of lactation (0–1 mo, 2–6 mo, 7+ months) can be found in Figure 1. *Streptococcus uberis* remained the dominant pathogen at all stages of lactation, which was isolated from $>35\%$ of samples, regardless of stage of lactation. No clear trends of increasing or decreasing prevalence during lactation were

evident for *Staph. aureus*, *E. coli*, or *Strep. dysgalactiae*. *Corynebacterium bovis* was the only pathogen that displayed a trend of increasing prevalence during later lactation, accounting for 0 samples (0.0%) in the first month of lactation, 2.2% of samples for 2 to 6 mo, and 3.2% in mo 7 or greater.

Pathogen profiles stratified by dairy region are shown in Table 1. The prevalence of *Strep. uberis* ranged from 33.3% in Gippsland to 43.8% in Western Victoria. *Staphylococcus aureus* prevalence ranged from 5.2% in Northern Victoria to 15.3% in Gippsland. Northern Victoria had the highest isolation rate of *E. coli* at 11.3%, which made it the second most likely pathogen to be isolated from clinical mastitis samples submitted from that region.

Herd-Level Pathogen Profiles for Clinical Mastitis. Herd-level pathogen profiles are summarized for herds ($n = 47$) that had 20 or more valid culture results in Table 2. Among these herds, the average number of submissions per herd was 50.4 (SD = 24.1, range from 20–161). In the average herd, gram-positive pathogens were isolated from 60.5% of samples (SD = 14.2%, range from 28.0–83.9%), with 37/47 (78.7%) herds having gram-positive pathogens isolated from at least 50% of samples (Figure 2). *Streptococcus uberis* was a common pathogen in many herds (mean = 39.9% of samples, SD = 14.7%, range from 4.3–67.5%). In the average herd, *Staph. aureus* and gram-negative pathogens accounted for 9.8% (SD = 10.3%, range 0.0–64.5%) and 11.3% (SD = 9.2%, range 0.0–47.1%) of samples, respectively.

Antimicrobial Susceptibility for Pathogens Isolated from Cows with Clinical Mastitis. The prevalence (95% CI) of AMR for clinical mastitis isolates is shown in Table 3. In general, AMR was uncommon among gram-positive pathogens. For *Strep. uberis* isolates, no AMR was identified for amoxicillin, cloxacillin, and penicillin. However, resistance was identified at a low prevalence against erythromycin [0.07 (i.e., 7%) 95% CI: 0.06, 0.09] and tetracycline (0.01, 95% CI: 0.01, 0.02). No clinical *Staph. aureus* isolates demonstrated resistance to cefuroxime, cloxacillin, penicillin-novobiocin, and trimethoprim-sulfamethoxazole. However, a small proportion of isolates were resistant to erythromycin (0.03, 95% CI: 0.02, 0.06), amoxicillin (0.02, 95% CI: 0.01, 0.04), and penicillin (0.02, 95% CI: 0.01, 0.04). Resistance among *Strep. dysgalactiae* isolates was identified against tetracycline (0.90, 95% CI: 0.85, 0.94) and erythromycin (0.07, 95% CI: 0.03, 0.12). *Escherichia coli* isolates demonstrated resistance to streptomycin and neomycin with prevalence at 0.36 (95% CI: 0.29, 0.43) and 0.28 (95% CI: 0.22, 0.34), respectively. *Klebsiella* spp. isolates demonstrated a

Figure 1. Histogram showing the percentage of the 6 most common culture results stratified by stage of lactation, grouped into the first month, mo 2 to 6, and >6 mo of lactation. The remaining culture outcomes are grouped together as Other*.

Figure 2. Histogram showing the herd-level prevalence of gram-positive isolates from cows with clinical mastitis ($n = 2,367$) on 47 southeastern Australian dairy farms collected between January 2011 and March 2012. The original data set consisted of 2,572 clinical mastitis samples from 65 herds, but samples from herds with <20 samples were excluded as they were expected to be an unreliable indicator of herd-level prevalence.

similar resistance profile to *E. coli* for most antimicrobials. *Serratia* spp. isolates had moderate to high levels of resistance to all antimicrobials evaluated, other than trimethoprim-sulfamethoxazole, where all isolates were susceptible. No AMR was identified for *Pasteurella* spp.

Subclinical Mastitis

Description of Enrolled Cows and Cases. A total of 1,079 composite milk samples were submitted

from cases of subclinical mastitis (i.e., cows with elevated SCC) at 47 different farms, but 7 records were excluded from all subsequent summaries as they were duplicate records, leaving 1,072 samples for analysis. These 1,072 samples came from 1,026 cows, with 42 cows having 2 samples and 2 cows having 3. Most of these animals had 2 to 3 samples at one time point, with only 12 cows having 2 samples at different time points. Eight of 47 farms submitted samples on 2 occasions (i.e., in 2 different months).

Pathogen Profile. Culture results from 1,072 samples of subclinical mastitis, stratified by region, are summarized in Table 4. A total of 425 out of 1,072 (39.6%) samples were excluded due to contamination. Of the remaining 647 valid results, 188 (29.1%) yielded “no growth.” *Staphylococcus aureus* was the organism most frequently isolated from subclinical mastitis samples ($n = 188$, 29.1%). *Streptococcus uberis* was the next most likely organism to be isolated ($n = 140$, 21.6%), whereas *E. coli* was only cultured on 10 occasions (1.5%). The prevalence of valid subclinical mastitis culture results for *Staph. aureus* was highest in Tasmania at 56.3%, and lowest in Northern Victoria at 21.0%. *Streptococcus uberis* isolates were lowest in Tasmania at 18.8% and highest in Gippsland at 24.3%.

Antimicrobial Susceptibility for Pathogens Isolated from Cows with Subclinical Mastitis. Prevalence of AMR for a selection of subclinical mastitis isolates including *E. coli*, *Serratia* spp., *Staph. aureus*, *Strep. dysgalactiae*, and *Strep. agalactiae* are

Table 2. Herd-level pathogen profile for clinical mastitis samples from 47¹ southeastern Australian dairy farms collected between January 2011 and March 2012

Item	Average	SD	Min	Max
Quarters with valid results (n)	50.4	24.1	20.0	161.0
No growth (%)	27.5	12.2	4.8	60.9
Gram-positive (%)	60.5	14.2	28.0	83.9
CNS (%)	0.7	1.3	0.0	4.5
<i>Corynebacterium bovis</i> (%)	1.6	2.4	0.0	12.0
<i>Listeria monocytogenes</i> (%)	0.1	0.6	0.0	2.9
<i>Nocardia</i> spp. (%)	1.7	2.1	0.0	10.7
<i>Staphylococcus aureus</i> (%)	9.8	10.3	0.0	64.5
<i>Streptococcus agalactiae</i> (%)	0.0	0.0	0.0	0.0
<i>Streptococcus dysgalactiae</i> (%)	6.1	6.1	0.0	26.5
<i>Streptococcus uberis</i> (%)	39.9	14.7	4.3	67.5
<i>Trueperella pyogenes</i> (%)	0.6	1.2	0.0	4.5
Gram-negative (%)	11.3	9.2	0.0	47.1
<i>Escherichia coli</i> (%)	8.9	8.7	0.0	47.1
<i>Klebsiella</i> spp. (%)	0.7	1.5	0.0	6.7
<i>Pasteurella</i> spp. (%)	0.7	1.6	0.0	7.7
<i>Pseudomonas aeruginosa</i> (%)	0.1	0.6	0.0	3.3
<i>Salmonella</i> spp. (%)	0.1	0.5	0.0	3.2
<i>Serratia</i> spp. (%)	0.7	1.1	0.0	4.4
Other bacteria (%)	0.3	0.8	0.0	4.2
<i>Candida</i> spp. or yeasts (%)	0.5	1.2	0.0	4.7

¹The original data set consisted of 2,572 valid results from clinical mastitis samples from 65 herds, but samples from herds with <20 samples were excluded ($n = 205$, 8.0%).

Table 3. Prevalence of antimicrobial resistance (95% CI) among pathogens isolated from clinical mastitis samples in southeastern Australian dairy cows

Antimicrobial	<i>Escherichia coli</i> (n = 213–216) ¹	<i>Klebsiella</i> spp. (n = 16–20)	<i>Pasteurella</i> spp. (n = 14–16)	<i>Serratia</i> spp. (n = 16–19)	<i>Staphylococcus aureus</i> (n = 271–272)	<i>Streptococcus dysgalactiae</i> (n = 159–165)	<i>Streptococcus uberis</i> (n = 975–1,008)
Amoxicillin	NT ²	1.00 (0.79, 1.00)	0.00 (0.00, 0.22)	NT	0.02 (0.01, 0.04)	0.01 (0.00, 0.03)	0.00 (0.00, 0.00)
Amoxicillin-clavulanate	0.04 (0.02, 0.08)	0.10 (0.01, 0.32)	0.00 (0.00, 0.23)	NT	NT	NT	NT
Cefuroxime	0.00 (0.00, 0.03)	0.00 (0.00, 0.17)	0.00 (0.00, 0.22)	NT	0.00 (0.00, 0.01)	0.01 (0.00, 0.03)	0.00 (0.00, 0.01)
Cloxacillin	NT	NT	NT	NT	0.00 (0.00, 0.01)	0.01 (0.00, 0.03)	0.00 (0.00, 0.00)
Erythromycin	NT	NT	NT	NT	0.03 (0.02, 0.06)	0.07 (0.03, 0.12)	0.07 (0.06, 0.09)
Neomycin	0.28 (0.22, 0.34)	0.32 (0.13, 0.57)	NT	0.16 (0.03, 0.40)	0.00 (0.00, 0.02)	NT	NT
Penicillin	NT	NT	0.00 (0.00, 0.21)	NT	0.02 (0.01, 0.04)	0.01 (0.00, 0.03)	0.00 (0.00, 0.00)
Penicillin-novobiocin	NT	NT	NT	NT	0.00 (0.00, 0.01)	0.01 (0.00, 0.03)	0.00 (0.00, 0.01)
Streptomycin	0.36 (0.29, 0.43)	0.21 (0.06, 0.46)	NT	0.28 (0.10, 0.53)	NT	NT	NT
Tetracycline	0.12 (0.08, 0.17)	0.00 (0.00, 0.17)	0.00 (0.00, 0.21)	0.72 (0.47, 0.90)	0.00 (0.00, 0.02)	0.90 (0.85, 0.94)	0.01 (0.01, 0.02)
Trimethoprim-sulfamethoxazole	0.08 (0.05, 0.12)	0.05 (0.00, 0.25)	0.00 (0.00, 0.22)	0.00 (0.00, 0.18)	0.00 (0.00, 0.01)	0.00 (0.00, 0.02)	0.00 (0.00, 0.01)

¹Resistance for some antimicrobials were not tested, resulting in a range in the number of tests for each pathogen-antimicrobial combination.²NT = not tested (or insufficient data). The 4 clinical *Strep. agalactiae* isolates were susceptible to all antimicrobials assayed.

shown in Table 5. *Staphylococcus aureus* isolates demonstrated some resistance against penicillin (0.15, 95% CI: 0.10, 0.21) and amoxicillin (0.14, 95% CI: 0.09, 0.20), whereas *Strep. uberis* isolates demonstrated resistance to erythromycin (0.07, 95% CI: 0.03, 0.12) and tetracycline (0.03, 95% CI: 0.01, 0.07). Antimicrobial resistance against tetracycline was common among *Strep. dysgalactiae* isolates (0.72, 95% CI: 0.53, 0.86).

Only a small number of gram-negative isolates were available for analysis, including *E. coli* (n = 9) and *Serratia* spp. (n = 4). For *E. coli*, AMR was identified against neomycin (0.44, 95% CI: 0.14, 0.79), streptomycin (0.44, 95% CI: 0.14, 0.79), tetracycline (0.11, 95% CI: 0.00, 0.48), and trimethoprim-sulfamethoxazole (0.11, 95% CI: 0.00, 0.48). *Serratia* spp. isolates demonstrated some resistance to antimicrobials other than trimethoprim-sulfamethoxazole, with AMR prevalence ranging from 0.25 to 1.00.

Comparison of AMR in Clinical and Subclinical Isolates

Effect estimates from 7 generalized linear mixed models describing the relationship between infection type (clinical vs subclinical) and risk of AMR are shown in Table 6. The risk of *Staph. aureus* demonstrating resistance to penicillin was 5.16 (95% CI: 1.68, 15.88) times higher in isolates collected from cows with subclinical mastitis than those from clinical isolates and 4.70 (95% CI: 1.49, 14.75) times higher for amoxicillin. The remaining models for *Staph. aureus* (erythromycin), *Strep. dysgalactiae* (erythromycin, tetracycline) and *Strep. uberis* (erythromycin, tetracycline) did not identify a significant association between infection type and resistance.

DISCUSSION

Streptococcus uberis Is the Most Common Mastitis-Causing Pathogen on Southeastern Australian Dairy Farms

The results from this study indicated that *Strep. uberis* was the most important mastitis-causing pathogen on southeastern Australian dairy farms (39.2% of samples). This finding is consistent with smaller surveys of dairy herds in the Gippsland region, which found that *Strep. uberis* was isolated from 22.7% (Watson et al., 1996) and 26.6% (Gunn et al., 1999) of clinical mastitis samples. This finding is also consistent with surveys conducted in pasture-based dairy herds in New Zealand, which found that *Strep. uberis* was isolated from 23.3% (Petrovski et al., 2009) and 33.8% (McDougall et al., 2007b) of clinical mastitis samples. Our find-

Table 4. Pathogen profile for subclinical mastitis samples from 47 southeastern Australian dairy farms collected between April 2011 and March 2012, stratified by region

Item	Gippsland (Victoria)		Northern Victoria		Tasmania		Western Victoria		All samples	
	n	%	n	%	n	%	n	%	n	%
Total samples submitted	316		348		47		361		1,072	
Contaminated	114	36.1	143	41.1	15	31.9	153	42.4	425	39.6
Total quarters with valid results	202	100.0	205	100.0	32	100.0	208	100.0	647	100.0
No growth	48	23.8	89	43.4	5	15.6	46	22.1	188	29.1
Growth	154	76.2	116	56.6	27	84.4	162	77.9	459	70.9
Gram-positive										
<i>Corynebacterium bovis</i>	11	5.4	10	4.9	1	3.1	12	5.8	34	5.3
<i>Nocardia</i> spp.	5	2.5	2	1.0	0	0.0	1	0.5	8	1.2
<i>Staphylococcus aureus</i>	57	28.2	43	21.0	18	56.3	70	33.7	188	29.1
<i>Streptococcus agalactiae</i>	0	0.0	0	0.0	1	3.1	0	0.0	1	0.2
<i>Streptococcus dysgalactiae</i>	22	10.9	4	2.0	1	3.1	9	4.3	36	5.6
<i>Streptococcus uberis</i>	49	24.3	43	21.0	6	18.8	42	20.2	140	21.6
CNS	3	1.5	2	1.0	0	0.0	14	6.7	19	2.9
Gram-negative										
<i>Escherichia coli</i>	2	1.0	2	1.0	0	0.0	6	2.9	10	1.5
<i>Pseudomonas aeruginosa</i>	1	0.5	1	0.5	0	0.0	4	1.9	6	0.9
<i>Serratia</i> spp.	1	0.5	1	0.5	0	0.0	2	1.0	4	0.6
Other bacteria	3	1.5	2	1.0	0	0.0	2	1.0	7	1.1
<i>Candida</i> spp. or yeasts	0	0.0	6	2.9	0	0.0	0	0.0	6	0.9

ings indicated that *Staph. aureus* (10.6% of samples) remains a relatively common cause of clinical mastitis in Australian dairy herds, although the proportion of samples associated with this pathogen was lower than in historical studies, which found higher proportions of *Staph. aureus* at 38.5% (Daniel et al., 1982) and 26.3% (Watson et al., 1996). This apparent reduction in prevalence is likely due to ongoing mastitis control strategies promoted through Dairy Australia's mastitis control program (Brightling et al., 2018).

"No growth" was a common outcome in this study, occurring in 27.5% of samples. However, this finding was lower than in 2 previous surveys of mastitis patho-

gens in pasture-fed dairy cattle where "no growth" was reported in 38.7% (Daniel et al., 1982) and 34.4% (Gunn et al., 1999) of samples, and slightly higher than another Australian study by Shum et al. (2009) where "no growth" was reported in 24.4% of samples. False-negative results (i.e., pathogens not being isolated in milk from an infected quarter) can be due to pathogens being killed by the inflammatory response within the mammary gland before sample collection (Erskine and Bartlett, 1993; Vangroenweghe et al., 2004) and due to unfavorable storage (Schukken et al., 1989) or culture conditions. In addition to these constraints, infections caused by *Mycoplasma* spp. cannot be readily identified

Table 5. Prevalence of antimicrobial resistance (95% CI) among pathogens isolated from subclinical mastitis samples in southeastern Australian dairy cows

Antimicrobial	<i>Escherichia coli</i> (n = 6–9) ¹	<i>Serratia</i> spp. (n = 4)	<i>Staphylococcus aureus</i> (n = 154–183)	<i>Streptococcus dysgalactiae</i> (n = 31–32)	<i>Streptococcus uberis</i> (n = 134–138)
Amoxicillin	NT ²	NT	0.14 (0.09, 0.20)	0.00 (0.00, 0.11)	0.00 (0.00, 0.03)
Amoxicillin-clavulanate	0.00 (0.00, 0.37)	NT	NT	NT	NT
Cefuroxime	0.00 (0.00, 0.46)	NT	0.00 (0.00, 0.02)	0.00 (0.00, 0.11)	0.00 (0.00, 0.03)
Cloxacillin	NT	NT	0.00 (0.00, 0.02)	0.00 (0.00, 0.11)	0.00 (0.00, 0.03)
Erythromycin	NT	NT	0.03 (0.01, 0.07)	0.03 (0.00, 0.16)	0.07 (0.03, 0.12)
Neomycin	0.44 (0.14, 0.79)	0.25 (0.01, 0.81)	0.00 (0.00, 0.02)	NT	NT
Penicillin	NT	NT	0.15 (0.10, 0.21)	0.00 (0.00, 0.11)	0.00 (0.00, 0.03)
Penicillin-novobiocin	NT	NT	0.00 (0.00, 0.02)	0.00 (0.00, 0.11)	0.00 (0.00, 0.03)
Streptomycin	0.44 (0.14, 0.79)	0.75 (0.19, 0.99)	NT	NT	NT
Tetracycline	0.11 (0.00, 0.48)	1.00 (0.40, 1.00)	0.00 (0.00, 0.02)	0.72 (0.53, 0.86)	0.03 (0.01, 0.07)
Trimethoprim- sulfamethoxazole	0.11 (0.00, 0.48)	0.00 (0.00, 0.60)	0.00 (0.00, 0.02)	0.00 (0.00, 0.11)	0.00 (0.00, 0.03)

¹Resistance for some antimicrobials were not tested, resulting in a range in the number of tests for each pathogen-antimicrobial combination.

²NT = not tested (or insufficient data).

Table 6. Results from 7 generalized linear mixed models¹ comparing antimicrobial resistance in bacteria isolated from cows with clinical and subclinical mastitis

Model	Pathogen	Antibiotic	Adjusted risk of resistance		Risk ratio ²	95% CI
			Clinical	Subclinical		
1	<i>Staphylococcus aureus</i>	Penicillin	0.01	0.07	5.16	1.68–15.88
2	<i>Staphylococcus aureus</i>	Amoxicillin	0.01	0.06	4.70	1.49–14.75
3	<i>Staphylococcus aureus</i>	Erythromycin	0.01	0.02	2.12	0.67–6.73
4	<i>Streptococcus dysgalactiae</i>	Erythromycin	0.05	0.02	0.37	0.04–3.08
5	<i>Streptococcus dysgalactiae</i>	Tetracycline	0.90	0.72	0.80	0.51–1.24
6	<i>Streptococcus uberis</i>	Erythromycin	0.06	0.04	0.75	0.36–1.54
7	<i>Streptococcus uberis</i>	Tetracycline	0.01	0.03	2.66	0.85–8.37

¹Models to compare clinical and subclinical samples, with random effects used to account for the clustering of samples within farms.

²The risk of resistance in subclinical samples divided by the risk in clinical isolates. For example, a risk ratio for model 1 indicates that the risk of penicillin resistance for *Staph. aureus* was 5.16 times higher in isolates collected from cows with subclinical mastitis, than cows with clinical mastitis.

using standard aerobic culture, the method used in this study. Therefore, it remains unknown what proportion of cases of clinical mastitis in Australian herds are due to *Mycoplasma* spp. infections.

The proportion of contaminated samples in the current study was relatively high, especially for subclinical isolates. This may be due to improper sampling methods by producers. However, it was also likely due to the use of a relatively strict definition for contamination (i.e., 2 or more isolates = contaminated). This is supported by surveys that have identified mixed infections in 2.7% (Olde Riekerink et al., 2008) and 6.6% (Shum et al., 2009) of clinical mastitis cases. A recent survey of late-lactation cows without clinical mastitis identified that 1.4% of quarters were infected with 2 pathogens (Rowe et al., 2019) and that 38.8% of cows had 2 or more infected quarters (Rowe et al., 2021). Therefore, it is likely that some “contaminated” composite samples from subclinical mastitis samples were mixed infections rather than contaminated. Consequently, omitting isolates from presumed contaminated samples could have resulted in the recruitment of isolates that differed from the actual pathogen profile in Australian herds, which could cause selection bias and reduce generalizability of our findings.

Pathogen Profiles Are Similar in Pastured-Based Herds in Australia and New Zealand

Our findings indicated that the pathogen profile for southeastern Australian dairy herds is likely to be similar to pasture-based herds in New Zealand, but different to herds managed in confinement systems in New South Wales (Shum et al., 2009), the United States (Lago et al., 2011), and Canada (Olde Riekerink et al., 2008), where gram-negative pathogens are much more common. This difference may be due to dietary factors

influencing the fecal shedding of coliforms in cows that are housed and fed a TMR (Tkalcic et al., 2000). Likewise, pasture-fed dairy cows are exposed to laneways and pastures that become heavily contaminated with *Strep. uberis* during wet conditions (Lopez-Benavides et al., 2007), which are common around the time of calving in pasture-fed dairy herds in southeastern Australia and New Zealand. One of the main strategies to manage environmental mastitis is the use of internal teat sealants at dry-off to prevent intramammary infection during the dry period (Runciman et al., 2010). Although 56.9% of participants in this survey reported the use of an internal teat sealant at dry-off, adoption of this nonantimicrobial mastitis control strategy has likely increased since this study was conducted.

Clinical Mastitis Pathogen Profiles Varied Among Herds

The profile of pathogens associated with clinical mastitis varied among herds (Table 2). For example, the percentage of samples from each herd that isolated *Staph. aureus* ranged from 0.0 to 64.5%, whereas the range for *E. coli* herd prevalence was 0.0 to 47.1%. This variability indicated that the “average” herd in this study was unlikely to be representative of all Australian herds. Therefore, we recommend that producers build their own pathogen profile by submitting samples to a veterinary diagnostic laboratory. Gram-positive pathogens were isolated from 60.5% of samples in the “average” herd in this study, with a range from 28.0 to 83.9%, with 37 out of 47 (78.7%) herds having gram-positive pathogens isolated from at least 50% of samples. This finding indicated that in the average herd, a selective treatment strategy, which typically reserves antimicrobial therapy for cases yielding gram-positive pathogens (Lago et al., 2011; McDougall et al., 2018; Fuenzalida

and Ruegg, 2019a,b), could theoretically reduce antimicrobial use by 39.5%, ranging from 16.1% (i.e., the herd with the largest proportion of cases caused by gram-positive pathogens) to 72.0%. This hypothesis requires further investigation because cases without a diagnosis (i.e., those not sampled or with inconclusive results due to contamination) are treated along with gram-positive cases. Furthermore, the herd-level pathogen profiles identified in this study were based on a variable number of samples from each herd, and diagnoses were obtained using laboratory-based aerobic culture, and not on-farm testing. Recent clinical trials in New Zealand, a region with similar pathogen profiles to those observed in this study, have found that selective treatment strategies based on rapid culture (McDougall et al., 2018) or a novel colorimetric test (Bates et al., 2020) reduced antimicrobial use by 25 and 24%, respectively, which is significantly less than the hypothetical number identified in this study. Given the increasing need to refine how antimicrobials are used on dairy farms, we believe more research is needed to evaluate the effect of implementing selective treatment strategies for clinical mastitis on antimicrobial use and cow health in Australian herds. Furthermore, partial budget analyses are needed to evaluate the cost-effectiveness for selective treatment strategies implemented under economic conditions seen on dairy farms in Australia, as has been done in other countries (Pinzón-Sánchez et al., 2011; Down et al., 2017).

Prevalence of AMR Was Low for Common Pathogens

In this study, pathogens that were commonly isolated from cows with clinical (Table 3) and subclinical (Table 5) mastitis (i.e., *Strep. uberis*, *Staph. aureus*, and *Strep. dysgalactiae*) were rarely resistant to antimicrobials that are commonly used for mastitis control in Australian dairy herds, which are listed in Supplemental Table S1 (<https://osf.io/dfq7p>; Taylor, 2021). Surveys of mastitis pathogens conducted in other countries have found similarly low (Bengtsson et al., 2009) or higher prevalence of AMR to the antimicrobials evaluated in this study among the same pathogens (Makovec and Ruegg, 2003; McDougall et al., 2014; Cheng et al., 2019). However, methods for identifying AMR vary among studies, making direct comparisons problematic. The findings from this study suggested that decades of antimicrobial use within the Australian dairy industry for mastitis control appear to have had little effect on AMR development. This conclusion is supported by a review by Oliver et al. (2011), which failed to find consistent evidence of an emerging trend of AMR among mastitis pathogens. However, our findings may not be

applicable in countries where AMR is more common in mastitis pathogens (Kalmus et al., 2011).

There were some instances in the current study where AMR was identified for some pathogen-antimicrobial combinations. For example, among both clinical and subclinical isolates of *E. coli*, *Klebsiella* spp., and *Serratia* spp., resistance was documented to streptomycin and neomycin to varying degrees. Antimicrobial susceptibility for these same antimicrobials were not evaluated against all pathogens, so it was not possible to determine if this resistance was evident across a broader range of pathogens.

For clinical and subclinical isolates of *Strep. dysgalactiae*, there was a high prevalence of resistance identified to tetracycline. This finding is consistent with studies conducted in New Zealand (Petrovski et al., 2011), Belgium, (Supré et al., 2014), and Canada (Cameron et al., 2016), where 88.8, 93.2, and 82.2% of isolates of *Strep. dysgalactiae*, respectively, were classified as resistant to tetracycline. Cameron et al. (2016) concluded that this is likely to be a common finding with this pathogen-antimicrobial combination due to the common use of tetracyclines to treat a range of infections of cattle (and humans), mobility of genetic determinants of tetracycline resistance between bacterial genera, and hosts and apparent persistence of tetracycline resistance in the absence of selection pressure. However, levels of tetracycline resistance in *Strep. uberis* identified in these 3 studies varied, with this study and the New Zealand study finding low levels of tetracycline resistance, and Cameron et al. (2016) finding very high levels of tetracycline resistance in both *Strep. uberis* and *Strep. dysgalactiae*. This requires further exploration in future studies, but may be due to variation in the proportion of this organism which is maintained in the environment, versus being cow-associated and thus exposed to systemic treatments with tetracycline used to treat other conditions (Nobrega et al., 2018a). *Streptococcus uberis* and *Strep. dysgalactiae* isolates in this study also demonstrated some resistance to erythromycin.

However, levels of penicillin and amoxicillin resistance in clinical *Staph. aureus* isolates in this study appear to be lower than levels reported by Daniel et al. (1982). The same trend of decreasing levels of penicillin resistance in this pathogen has been noted in New Zealand (McDougall et al., 2014) and the United States (Pol and Ruegg, 2007). The absence of high prevalence of AMR among *Staph. aureus* isolates in Australian dairy cattle is further supported by a study conducted in 2013 that found no evidence of methicillin resistance in 203 *Staph. aureus* isolates from mastitis submissions to 22 Australian veterinary diagnostic laboratories (Worthing et al., 2018).

The low AMR prevalence among common pathogens suggests that transfer of AMR from mastitis pathogens to humans under Australian conditions is unlikely. However, it should be noted that microorganisms other than recognized mastitis pathogens (e.g., those from the gastrointestinal tract) can also contaminate milk via the teat surface at milking, or enter the udder via environmental contamination, and may be potential sources of AMR. Another limitation is that our study did not focus on AMR pathogens that are commonly a problem in public health (e.g., methicillin-resistant *Staph. aureus*, extended spectrum β -lactamase producing *Enterobacteriaceae*). Therefore, more research is needed in Australian dairy herds to investigate the prevalence of AMR in bulk milk.

AMR and Antimicrobial Therapy for Clinical Mastitis

The predominance of gram-positive pathogens with low AMR prevalence in this study indicated that treatment outcomes for clinical mastitis may be similar among the products currently registered for mastitis treatment in Australia, assuming that AMR defined with human breakpoints is correlated with the outcome of antimicrobial therapy. This hypothesis is supported by several clinical trials conducted in New Zealand (where pathogen profiles are similar to those in Australia), showing similar responses to treatment (bacteriological cures, clinical cures) among antimicrobial treatments (McDougall, 1998, 2003; McDougall et al., 2007a; Bryan et al., 2016). In our opinion, antimicrobial stewardship could be improved on Australian dairy farms through the use of narrow-spectrum products (i.e., those targeting gram-positive pathogens only) that are administered via the intramammary route. Many factors other than antimicrobial susceptibility have been demonstrated to affect the probability of an infection curing after antimicrobial therapy, such as the ability of the pathogen to evade the immune response of the host, the chronicity of the infection (Melchior et al., 2006), and the duration of antimicrobial therapy (Truchetti et al., 2014).

***Staphylococcus aureus* Isolates from Subclinical Mastitis Were More Likely to Have Resistance Against Penicillin and Amoxicillin Than Clinical Isolates**

One interesting finding from this study was that *Staph. aureus* isolates from subclinical mastitis samples were 5.16 and 4.70 times more likely to have AMR for penicillin (0.07 vs. 0.01) and amoxicillin (0.06 vs. 0.01), respectively. Studies have demonstrated that antimicro-

bial therapy is less successful at clearing IMI caused by *Staph. aureus* strains with mechanisms for AMR (Barkema et al., 2006). Therefore, it is plausible that the use of antimicrobial therapy during lactation and at dry-off may apply selection pressure to existing subclinical IMI within the herd, such that resistant strains of *Staph. aureus* are more likely to establish chronic infections and remain prevalent in the herd. The underlying mechanisms for this require further evaluation using data sets with sufficient detail to identify infection patterns, such as chronicity and responses to therapy. Furthermore, additional studies using molecular methods are needed to compare the mechanisms for AMR between clinical and subclinical isolates.

Strengths and Limitations

A major strength of this study was the large number of cows sampled ($n = 2,651$) from a large number of herds ($n = 65$) across multiple regions ($n = 4$) in southeastern Australia, which is where 75% of the Australian dairy industry is concentrated. A limitation was the large time lag between the time of the fieldwork (2011–2012) and publication. We had not originally planned to publish this research due to finding similar pathogen profiles of earlier Australian studies (the primary objective of this study). However, we were recently motivated to do so by the recommendation to conduct AMR surveillance (WHO-OIE, 2016) and the opportunity to contribute a baseline to compare AMR in Australian dairy herds in the future. Given the time lag, it is possible that pathogen profiles and AMR patterns in Australian dairy cows have changed. Future studies are required, and the results from this survey will be useful to guide their design. However, recent studies conducted in New Zealand have found similar pathogen profiles (McDougall et al., 2018, 2019), suggesting that the findings from this study may still be representative for many herds in Australia.

Laboratory methods have also changed during this time. For example, isolates in the current study were identified using biochemical tests, such as catalase, coagulase, and Lancefield group testing, instead of MALDI-TOF, which has replaced the use of biochemical tests in many udder health laboratories. Therefore, it is possible that *Enterococcus*, *Lactococcus*, and *Aerococcus* species were misclassified as *Streptococcus* spp. (Murray, 2010). Furthermore, disk diffusion methodology was used to measure AMR, which is now more commonly evaluated using broth dilution methods. Disk diffusion provides good agreement with broth microdilution on classification of isolates of mastitis pathogens as susceptible or resistant (Saini et al., 2011), but MIC data

allows more detailed monitoring of trends in AMR in bacterial populations over time through movement in the distribution of minimum inhibitory concentrations.

Panels of antimicrobials assayed for resistance varied among pathogens, as was the protocol at the laboratory. This limited our ability to make comparisons across pathogens for some antimicrobials. Another important limitation was that herds and cows were recruited by convenience sampling, rather than a probability sampling method. Therefore, it was possible for individual herds to submit more samples than others, which could bias the final estimates of pathogen prevalence. Furthermore, the inclusion criteria for samples with sub-clinical mastitis were broad, rather than samples being collected randomly from all eligible cows. Therefore, cows were likely enrolled according to the preferences of the producer, which may have favored selection of cows with a specific SCC dynamic (e.g., cows with recent increases in SCC or cows with chronically elevated SCC). Furthermore, samples were collected by farm workers rather than trained personnel, which is likely why the rate of contamination in this study was relatively high. Given that contamination status was unlikely to be associated with pathogen type, we doubt that the exclusion of contaminated samples from our data set caused selection bias.

CONCLUSIONS

The most common bacteria causing clinical mastitis in dairy herds in southeastern Australia is likely *Strep. uberis*, whereas *Staph. aureus* is likely to be the most common cause of subclinical mastitis. Despite decades of antimicrobial use to control these organisms, AMR appears to be uncommon.

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ORCIDS

- R. Dyson <https://orcid.org/0000-0002-7537-2699>
 N. Charman <https://orcid.org/0000-0001-8607-8789>
 A. Hodge <https://orcid.org/0000-0002-1584-5589>
 S. M. Rowe <https://orcid.org/0000-0001-8336-6523>
 L. F. Taylor <https://orcid.org/0000-0002-6065-7202>