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ORIGINAL ARTICLE

Prevalence and antimicrobial resistance of MRSA across different pig age groups in an intensive pig production system in Australia

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Abstract

This observational study aimed to determine MRSA prevalence using strain-specific real-time PCR at the pig level, stratified by age groupings, within a pig enterprise. A total of 658 samples were collected from individual pigs (n = 618) and the piggery environment (n = 40), distributed amongst five different pig age groups. Presumptive MRSA isolates were confirmed by the presence of *mecA*, and MALDI-TOF was performed for species verification. All isolates were tested against 18 different antimicrobials. MRSA was isolated from 75.2% (95% CI 71.8–78.6) of samples collected from pigs, and 71% of the MRSA isolates from this source were identified as community-associated (CA)-MRSA ST93, while the remainder were livestock-associated (LA)-MRSA ST398. Amongst environmental isolates, 80% (CI 64.3–95.7) were ST93 and the remainder ST398. All MRSA isolates from pigs and the environment were susceptible to ciprofloxacin, gentamicin, linezolid, mupirocin, rifampicin, sulfamethoxazole–trimethoprim, teicoplanin and vancomycin. Phenotypic rates of resistance were penicillin (100%), clindamycin (97.6%), erythromycin (96.3%), ceftiofur (93.7%), chloramphenicol (81.2%), tetracycline (63.1%) and amoxicillin–clavulanate (63.9%). A low prevalence of resistance (9.2%) was observed against neomycin and quinupristin–dalfopristin. The probability of MRSA carriage in dry sows (42.2%) was found to be significantly lower (p < .001) when compared to other age groups: farrowing sows (76.8%, RR1.82), weaners (97.8%, RR 2.32), growers (94.2%, RR 2.23) and finishers (98.3%, RR 2.33). Amongst different production age groups, a significant difference was also found in antimicrobial resistance for amoxicillin–clavulanate, neomycin, chloramphenicol and tetracycline. Using the RT-PCR assay adopted in this study, filtering of highly prevalent ST93 and non-ST93 isolates was performed at high throughput and low cost. In conclusion, this study found that weaner pigs presented a higher risk for CA-MRSA and antimicrobial resistance compared to other age groups. These findings have major implications for how investigations of MRSA outbreaks should be approached under the One-Health context.

KEYWORDS
antimicrobial resistance, CA-MRSA, LA-MRSA, livestock, methicillin-resistant *Staphylococcus aureus*, one health, pigs, public health, ST398, ST93

1 | INTRODUCTION

Staphylococcus aureus is a component of the normal flora of the integument of humans, pigs and other livestock species, with MRSA isolates typically carried by pigs and other livestock referred to as livestock-associated (LA) MRSA. The organism behaves as an opportunistic pathogen in most hosts. While it is of limited clinical importance in pigs, in humans it is a frequent cause of skin and soft tissue infections with potential to progress to fatal bacteraemia. Methicillin-resistant forms of *Staphylococcus aureus* (MRSA) have become increasingly common in humans and are regarded as a serious threat to the health of individuals and the community (Uhlemann, Otto, Lowy, & DeLeo, 2014). There are several distinct lineages of MRSA that colonize and cause clinical infections in both humans and some livestock species (Coombs et al., 2016; Silva et al., 2014). Molecular typing is a key step for understanding the epidemiology of MRSA in scenarios where multiple host species are present. A range of methods such as multilocus sequence typing (MLST; Saunders & Holmes, 2014), pulse field gel electrophoresis (PFGE; Golding, Campbell, Spreitzer, & Chui, 2015), staphylococcal protein A (*spa*) typing (Narukawa, Yasuoka, Note , & Funada, 2009) and direct repeat unit (*dru*) typing (Goering, Morrison, Al-Doori, Edwards, & Gemmell, 2008) exist and can be used for typing of isolates. The MLST and *spa* typing approaches are considered as 'gold standard' for *S. aureus* typing due to their harmonized protocols and nomenclature that allows high inter-laboratory reproducibility and comparison of data across all host populations (Kinross et al., 2017; Williamson, Heffernan, & Nimmo, 2015).

Although both methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) forms occur, the recent literature has been dominated by reports of the latter (Crombé et al., 2013; Yan et al., 2014). Additionally, human infections caused by MRSA are more difficult to treat due to fewer options for effective antimicrobials. Sequence type (ST) 398 is the most frequently documented LA-MRSA in pigs in Europe. In the USA, ST398 and ST5 are the most frequently reported LA-MRSA (Frana et al., 2013; Narvaez-Bravo et al., 2016), and in Asia, ST9 appears to be the predominant strain of LA-MRSA isolated from pigs and pig farmers, although ST398 is also present (Fang, Chiang, & Huang, 2014, Ye et al., 2016). ST398 comprises different *spa* types such as t011, t034 and t108 (Köck et al., 2013). ST398 is reported to be resistant to fewer non-beta-lactam antimicrobials compared to non-ST398 strains (Mutters et al., 2016), although greater resistance to macrolides, lincosamides and tetracycline is reported (Conceição, De Lencastre, & Aires-De-Sousa, 2017). In Australia, ST93 is the most prevalent community-associated (CA-MRSA) strain and is the second most common strain to cause infection in hospitals (Brennan et al., 2013; Coombs, Pearson, & Robinson, 2015; Munckhof et al., 2009). This highly virulence ST was first reported in Australian pigs in 2017 (Sahibzada et al., 2017). Although ST93 is beta-lactam resistant, it is generally susceptible to most other antimicrobial classes (Coombs et al., 2009).

Impacts

- Community-acquired MRSA infections occur in pigs.
- High prevalence of asymptomatic colonization of pigs with MRSA (>70%) exacerbates the risk of zoonotic transmission to farmworkers.
- Age class of animal needs to be considered as a design factor in studies aiming to quantify the occurrence of MRSA in pig populations accurately.
- Pig herds can be concurrently colonized with multiple sequence types of MRSA including those that commonly colonize humans.
- Multi-clone MRSA infection of pig herds necessitates subjecting an adequate number of isolates to sequence typing in outbreak investigations and surveillance.
- Use of sequence type differentiating real-time PCR is a cost-effective method in typing both livestock and community-associated ST93 and ST398 MRSA.

Recently, a high rate of carriage of MRSA was identified at an Australian pig enterprise experiencing an ongoing MRSA outbreak amongst its human workforce, as identified by the relevant state public health authority; however, no information is accessible about the actual clinical presentations. The microbiological and genomic analysis of a small (n = 37) number of isolates revealed two MRSA strains, ST398 and ST93, to be present in pigs, humans and the environment.

In work reported here, the sampling and microbiological evaluation is expanded to characterize the epidemiology of MRSA in the affected herd. Thus, the current study represents an expansion of the initial investigation by Sahibzada et al. (2017) with a large-scale application of real-time PCR assays to examine the prevalence of specific sequence types of MRSA and their distribution amongst different age groups of pigs across the enterprise. Based on initial results identifying that working with specific age groups of pigs was a risk factor for MRSA carriage among piggery workers (Sahibzada et al., 2017), we hypothesized that the prevalence of MRSA carriage and resistance profile would be different across pig production age groups.

2 | METHODS

2.1 | Farm description and sampling

The affected pig enterprise had two sites referred to herein as site A and site B. The two sites were geographically separated by ~40 km, with animal movement and exchange of workers common between both sites. The age-class distribution of pigs was similar on both sites, and the number of animals varied between 1,000 and 3,000 pigs per shed. All buildings were naturally ventilated where pigs were housed in separate sheds based on their age group and were classed as dry sows (breeding stock), farrowing sows, weaners, growers and finishers. Site A had seven sheds: two housed dry sows, one farrowing sows, one weaners, two growers and one finishers. Site B had a total of 13 sheds: four housed dry sows, four farrowing sows, two weaners, two growers and one finishers. To detect MRSA carriage in pigs with an unknown prevalence (set at 50%) assuming 90% test sensitivity and specificity with 95% confidence level and 5% desired precision, the minimum number of samples were identified to be 601. We collected a total of 618 nasal swabs in pigs on both sites. The sampling for this cross-sectional study at each site was carried out over a two-day period, with site A visited in May and site B in August 2015. No information on antimicrobial use in the study populations was obtained.

In the previous study, different sampling techniques were used on each site for bacterial isolation, as explained in Sahibzada et al. (2017). In summary, nasal swabs were collected using a convenient selection procedure from pigs in their respective pens. A pooled sampling method was used on site A, whereas individual animal samples were processed in the laboratory for site B. In the current study, a common technique was used on both sites for MRSA sampling and bacterial isolation for the sake of consistency in results. Therefore, the entire set of individual pig samples (n = 408) collected during the initial investigation from site B were included. At site B, 30 samples were collected in each shed except for a farrowing sow shed where 48 samples were collected as this particular farrowing sow shed had eight different farrowing rooms, so nasal swabs from six pigs were collected in each room resulting in 48 swabs. For site A, the swabs were collected in duplicate from 60 individual animals in seven different sheds. One set of swabs was analysed in the laboratory within 24 hr of the collection while the second set of samples was stored at −80°C. Later on, a total of 30 samples out of the 60 nasal swabs were systematically chosen (samples with odd numbers) from each shed, resulting in 210 samples for site A. In all sheds, the pigs were randomly chosen, and animals that had been sampled were identified with a coloured marker to avoid re-sampling. In the farrowing sheds, a sow from a randomly selected crate was swabbed, and at the same time, nasal swabs were collected from a randomly chosen piglet in the same crate.

2.2 | Laboratory analysis

MRSA was isolated using the method previously described by Sahibzada et al. (2017). In brief, pig samples were pre-enriched in Mueller–Hinton broth containing 6.5% sodium chloride for 24 hr at 37°C. The five environmental samples per shed were pooled in 50 ml of the pre-enriched broth. Afterwards, 1 ml of broth from the pre-enriched culture was inoculated into 9 ml Tryptone Soy Broth supplemented with 3.5 mg/L cefoxitin and 75 mg/L aztreonam and incubated overnight at 37°C. Subsequently, a loopful was streaked onto chromogenic MRSA agar and incubated for 24 hr at 37°C. Ultimately, presumptive MRSA colonies were transferred on to sheep blood agar plates, and a latex agglutination test was performed with Staphylase (Oxoid™) kit. *Staphylococcus aureus* ATCC 29,213 was used as the reference strain. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was also performed, as per manufacturers instruction, on all isolates to confirm the *Staphylococcus aureus* species identification.

Confirmed *Staphylococcus aureus* isolates with presumptive methicillin resistance were subjected to antimicrobial susceptibility testing by the disc diffusion method following the Clinical and Laboratory Standards Institute (CLSI) protocols (CLSI, 2013, 2018) and involved the following 18 antimicrobials: amoxicillin–clavulanate, ceftiofur, cefoxitin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, linezolid, mupirocin, neomycin, penicillin, quinupristin–dalfopristin, rifampin, tetracycline, teicoplanin, sulfamethoxazole–trimethoprim and vancomycin.

2.3 | Molecular typing

2.3.1 | DNA extraction

DNA was extracted using 6% chelex as previously described by Abraham, Chin, Brouwers, Zhang, and Chapman (2012) with a minor modification. Briefly, a loop full of bacteria taken from freshly cultured blood agar was suspended in 100 µl of molecular grade water and centrifuged at 2,040 × g for 2 min. The supernatant was removed, followed by addition of 100 µl of 6% chelex matrix. Subsequently, the chelex mixture with bacterial colonies was incubated at 56°C for 20 min and then 100°C for 5 min. The mixture was centrifuged at 2040 xg for five minutes at room temperature, and the supernatant was used in the PCR reaction. DNA quantification was performed using a Qubit HS DNA assay kit (Invitrogen) on a Qubit-3.0 Fluorometer.

2.3.2 | Real-time PCR

All MRSA isolates were screened for *mecA* and PVL (Panton–Valentine Leukocidin) genes by singleplex real-time polymerase chain reaction (RT-PCR). A lineage-specific singleplex RT-PCR was also used for detection of ST93 and ST398. A list of primers and probes used in this study is given in Table 1. TaqMan® Fast Master Mix probe-based PCR reagents were used for detection of *mecA* genes and strain type ST398 whereas SYBR® Green reagents assay-based PCR were used for the detection of PVL gene and strain type ST93. The design of the primers for the ST93 assay was adapted from Pang et al. (2012). The

assay consists of two primers aroE252G and aroE252T which differ by one nucleotide at the 3′ end. The ST93 specific primer aroE252G primer generates a CT difference of ten cycles upon a comparison between amplification curves to non-ST93 isolates. The MRSA isolates that were previously characterized by whole-genome sequencing for strain typing and detection of PVL genes by Sahibzada et al. (2017) were used as a control for comparison.

The RT-PCR SNP assay was made to a total volume of 10 µl and was amplified on the QuantStudio™ 6 Flex Real-Time PCR system using a 96-well plate template set-up. For each reaction, the assay contained forward and reverse primer at a concentration of 0.5 µM, 2 µl of nuclease-free water, 5 µl master mix (PowerUp SYBR™ Green Master Mix) and 2 µl of DNA template. The ST398 and *mecA* assays were probe-based and consisted of primers at a final concentration of 0.9 µM, 1 µl of nuclease-free water, 5 µl master mix (TaqMan® Fast Advanced Master Mix), probe at a final concentration of 0.2 µM and 2 µl of DNA template.

The PCR conditions for the probe-based assay consisted of an initial hold stage of 2 min at 50°C and 20 s at 95°C (polymerase activation), 40 cycles with one second at 95°C (denature) followed by 20 s at 60°C (annealing/extension).

The PCR condition for SYBR green assay was as follows. The initial hold stage was set for 2 min at 50°C followed by another 2 min at 95°C (for enzyme activation). The PCR stage was set up for 40 cycles of three-temperature cycling consisting of 95°C for 15 s (for denaturation), 50°C for 15 s and 72°C for one minute (for annealing and polymerization). The third stage (melt-curve) was used to confirm the specificity of the amplification products. The melt-curve stage was set at 90°C for 15 s, 60°C for 1 min and 95°C for 15 s.

2.3.3 | *Staphylococcus Protein A* typing

A subset of 30 MRSA isolates from pigs (n = 24) and environment (n = 6) that were previously sequenced using whole-genome sequencing (Sahibzada et al., 2017) were characterized by *spa* gene typing as previously described (Harmsen et al., 2003) using whole gene sequence data. The *spa* type was determined using the spaTyper tool through the Centre for Genomic Epidemiology (Camacho et al., 2009).

2.4 | Statistical analysis

All analyses were performed using the R statistical package version 3.6.2 (R Core Team, 2019). Data on inhibition zone size for each drug were converted to a dichotomous classification of resistant (R) or susceptible (S), as recommended in the CLSI documents M100-S24 (CLSI, 2018) and VET01-S2 (CLSI, 2013). All intermediate resistance isolates were considered as susceptible. Isolates resistant to at least one beta-lactam and two non-beta-lactam antimicrobial classes were categorized as multidrug-resistant (MDR). The Kruskal–Wallis test was used to identify significant differences in median number of MDR isolates between different pig age groups using MDR as a dependent variable with integers (1–7) and shed as an independent variable with five-factor levels. Generalized linear models (GLMs) were used to assess associations between MRSA carriage (modelled as a dependent binary variable) and pig age groups (independent variable). The association between antimicrobial resistance and pig age group was assessed by including each phenotype as a dependent variable and using separate univariable GLMs. The risk ratio (RR) and confidence intervals (CI) for each antimicrobial resistance were calculated from GLM models. All comparisons of proportions and 95% confidence intervals for proportions were calculated using the proportion test in R. Plotting was done in R using the package ggplot2 for a graphical display (Wickham et al., 2019).

2.5 | Ethics approval

This study was based upon voluntary participation of the producer. The study was approved by the Charles Sturt University Human

TABLE 1 List of the primers and probes used for the detection of *mecA*, PVL, ST398 and ST93 amongst MRSA isolates collected on two sites of a piggery in Australia

Target gene	Primer and probe	Sequence	Reference
mecA	MECA-F	5′-TGGTATGTGGAAGTTAGATTGGGAT	Nakagawa et al. (2005)
	MECA-R	5′- CTAATCTCATATGTGTTCCTGTATTGGC	
	MECA-P1 (FAM)	5′- TTCCAGGAATGCAGAAAGACCAAAGCA	
pvl	PVL-F	5′- AAG GCT CAG GAG ATA CAA GTG	This study
	PVL-R	5′- TCA CTT CAT ATT TAA CTG TGT AAT TTC	
ST398	tmST398-F	5′- CATTCATCACACGTATATTCTATAGGTTCC	van Meurs et al. (2013)
	tmST398-R	5′- TAAGAAATTCTGTTATTAATTCAGATGGTCA	
	tmST398 (FAM)	5′- ACCGCAATTCATACTGAC	
ST93	aroE252-F	5′- ACCTGCGCCCAAAATTAAAA	Modified from Huygens et al. (2006)
	aroE252G-R†	5′- GGTATAATACAGATGGTATCGGTTATGTG	
	aroE252T-R§	5′- GGTATAATACAGATGGTATCGGTTATGTT	

†Primer used for detection of ST93.

§Primer used for detection of non-ST93.

Research Ethics Committee (Protocol number 2015/016) and Animal Care and Ethics Committee (Protocol number 14/096).

3 | RESULTS

3.1 | Overall MRSA prevalence

Overall, MRSA was isolated from 490 out of 658 samples from pigs and the environment. In pigs, a prevalence of 75.2% (465/618; 95% CI 71.8–8.6) was found across both sites. There was no significant difference (p = .27) in the proportion of MRSA-positive pigs between sites; there being 77.6% (163/210; 95% CI 71.9–83.2) positive on site A and 74.0% (302/408; 95% CI 69.8–78.3) on site B. Using RT-PCR, most of the pig MRSA isolates identified as CA-MRSA ST93 (71%; CI 67.2–75.5) while the remainder were LA-MRSA ST398 (29%; CI 24.4–32.7). The environmental swabs collected in different sheds returned positive results amongst all pig age groups. Amongst environmental isolates, only 20% (CI 4.3–35.7) were typed as ST398 and, 80% (CI 64.3–95.7) were ST93. Only one isolate from site A could not be typed using the methods outlined in this study but was identified as ST30 using whole-genome sequencing. Most of the ST93 (68.9%) isolated from pigs and environment were positive for the PVL encoded genes while all ST398 were negative. No significant difference (p = .5) was found between sites for PVL carriage amongst ST93. A higher proportion of ST93 (n = 130, 79.8%) was found on site A compared to site B (n = 201, 66.6%). Using the univariable model the risk ratio (RR) of ST93 carriage amongst pig isolates was estimated to be significantly higher for site A compared to site B (RR 1.96, CI 1.26–3.11, p = .003).

Four different *spa* types were identified amongst 30 selected MRSA isolates. The *spa* types were t202 (n = 12), t011 (n = 8), t2510 (n = 2), t8100 (n = 2) and six isolates containing *spa*-repeat patterns not associated to any known *spa* types, and could be due to sequencing quality within the spa region or new *spa* types. All unknown *spa* types belonged to ST93, the *spa* type t202 was the only known spa type related to ST93, and the remainder were associated with ST398. The MLST typing performed on the 25 environmental isolates identified twenty ST93 and five ST398.

3.2 | MRSA prevalence amongst different pig production age groups

MRSA was isolated from all pig age groups during the outbreak and shed level prevalence estimates ranged from 42.0% to 98.0% on both sites (Figure S1). The distribution and proportion of overall MRSA and the strain carriage across five different age groups are shown in Figure 1. A significant association between MRSA prevalence and pig age group was identified (p < .001). Overall, for both sites, the risk for MRSA carriage was lower in dry sows when compared with farrowing sows (RR 1.82, CI 1.58–2.10), weaners (RR 2.32, CI 1.99–2.7), growers (RR 2.23, CI 1.93–2.58) and finishers (RR 2.33, CI 1.96–2.76). An association between pig age group and carriage of MRSA strain type was also assessed. In comparison with the weaner group (within which 89% of the MRSA was typed as ST93), a significantly (p < .001) lower risk of ST93 carriage was found amongst dry sows (60%, RR 0.68, CI 0.55–0.82), farrowing sows (67%, (RR 0.75, CI 0.64–0.89), growers (69%, (RR 0.78, CI 0.66–0.92) and finishers (75%, (RR 0.84, CI 0.69–1.02). Within the farrowing group, there was no significant difference (p = .38) of MRSA carriage between the farrowing sows and piglets. No statistical difference was noted between sites with respect to overall MRSA carriage and strain carriage in different age groups (Figure S1).

3.3 | Overall antimicrobial resistance profile

None of the 490 MRSA isolates were resistant to ciprofloxacin, gentamicin, linezolid, mupirocin, rifampicin, trimethoprim/sulfamethoxazole, teicoplanin or vancomycin. A low frequency of resistance was identified to neomycin (9.1% CI 6.5–11.7) and quinupristin–dalfopristin (9.3% CI 6.7–11.9). Two-thirds of the MRSA isolates were resistant to amoxicillin–clavulanate (63.8% CI 59.5–68.0) and tetracycline (63.8% CI 59.5–68.0). Chloramphenicol resistance was observed in 80.9% (CI 77.4–84.4) of the isolates. Most of the MRSA isolates were resistant to ceftiofur (93.6% CI 91.4–95.8), erythromycin (96.5% CI, 94.8–98.1), clindamycin (97.7% CI 96.4–99.0) and penicillin (100%). Data regarding antimicrobial resistance and strain carriage among different pig age groups is provided in Table S1.

The proportion of resistance amongst ST398 and ST93 is plotted in Figure 2. A significantly greater proportion of resistance was observed for ST93 in comparison with ST398 against amoxicillin–clavulanate (RR 1.51, CI 1.28–1.79, p < .001), ceftiofur (RR 1.17, CI 1.11–1.24, p < .001), chloramphenicol (RR 2.88, CI 2.53–3.31, p < .001),

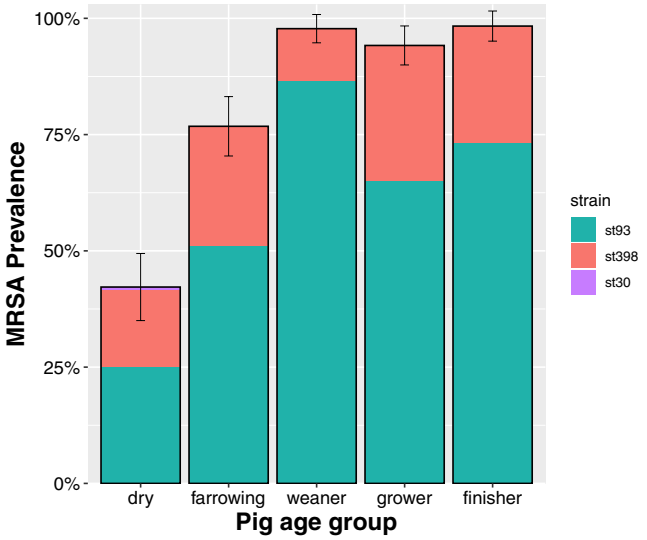

FIGURE 1 Distribution of the origin of MRSA pig isolates (n = 465) during an MRSA outbreak in piggery workers across different pig age groups and broken down by sequence type. Error bars depict 95% confidence intervals for overall MRSA prevalence

erythromycin (RR 1.05, CI 1.01–1.09, p = .03) and neomycin (RR 2.54, CI 1.2–6.37, p = .03). In contrast, a lower proportion of resistance was observed against quinupristin–dalfopristin (RR 0.07, CI 0.03–0.14, p < .001), and tetracyclines (RR 0.51, CI 0.44–0.58, p < .001) for ST93 compared with ST398. No significant difference was found for the anti-biogram profile between pig and environmental isolates (shown in Figure S2). The distribution of antimicrobial phenotype amongst MRSA isolates for both sites is displayed in Figure S3.

The majority of MRSA isolates collected in this study were observed to exhibit MDR. A total of 11 (2.4%) isolates were resistant to one or two non-beta-lactam antimicrobials, 267 (54.5%) to three, 128 (26.1%) to four and 79 (16.1%) to five, and four isolates (0.8%) were resistant to six of the non-beta-lactam drugs in the test panel. Eleven different resistance patterns to multiple antimicrobials were obtained among MRSA isolates as shown in Table 2. A significant difference (p < .001) was found between MDR pattern and pig age group. On average, weaner isolates were more likely (p < .001) to be resistant to a larger number of non-beta-lactam antimicrobials (median = 4, range 1–6) compared to the other production age groups. Amongst ST93 isolates, the most common pattern of multiple resistance (50.1%) to non-beta-lactam group was a combination of chloramphenicol, clindamycin and erythromycin. The majority of ST398 isolates (60.1%) were resistant to a combination of tetracycline, clindamycin and erythromycin.

Amongst different production age groups, a significant difference (p < .001) in antimicrobial resistance was found for amoxicillin–clavulanate, chloramphenicol, neomycin and tetracycline. Weaners, in comparison with other age groups, presented a higher risk for exhibiting resistance against these antimicrobials as shown in Table 3.

4 | DISCUSSION

This is the first comprehensive study describing the distribution and frequency of MRSA prevalence, including different strain carriage and antimicrobial resistance in the range of pig age groups present in an Australian pig herd experiencing an MRSA outbreak among piggery workers. Extensive sampling of pigs at all ages of production at a single pig enterprise under the same management system provided estimates of how MRSA carriage can vary due to their production age. The work also provides a unique profile of the behaviour in pigs of a CA-MRSA commonly isolated from humans where it causes virulent disease. The overall prevalence of MRSA carriage in pigs within this affected herd was just over 75%, which is consistent with reports from European and US studies, which have identified up to 85% MRSA carriage in pigs although typically only report LA-MRSA (Dewaele et al., 2011; Morcillo et al., 2015; Smith et al., 2009). In Australia, MRSA in pigs was first reported in 2014 (Groves et al., 2014), where 324 samples were collected from five different commercial pig farms and feral population, resulting in only three samples from a single farm being MRSA-positive (and further typed as ST398). Most of the MRSA isolates found in the current study (71.6%) were typed as ST93, a predominant CA-MRSA in

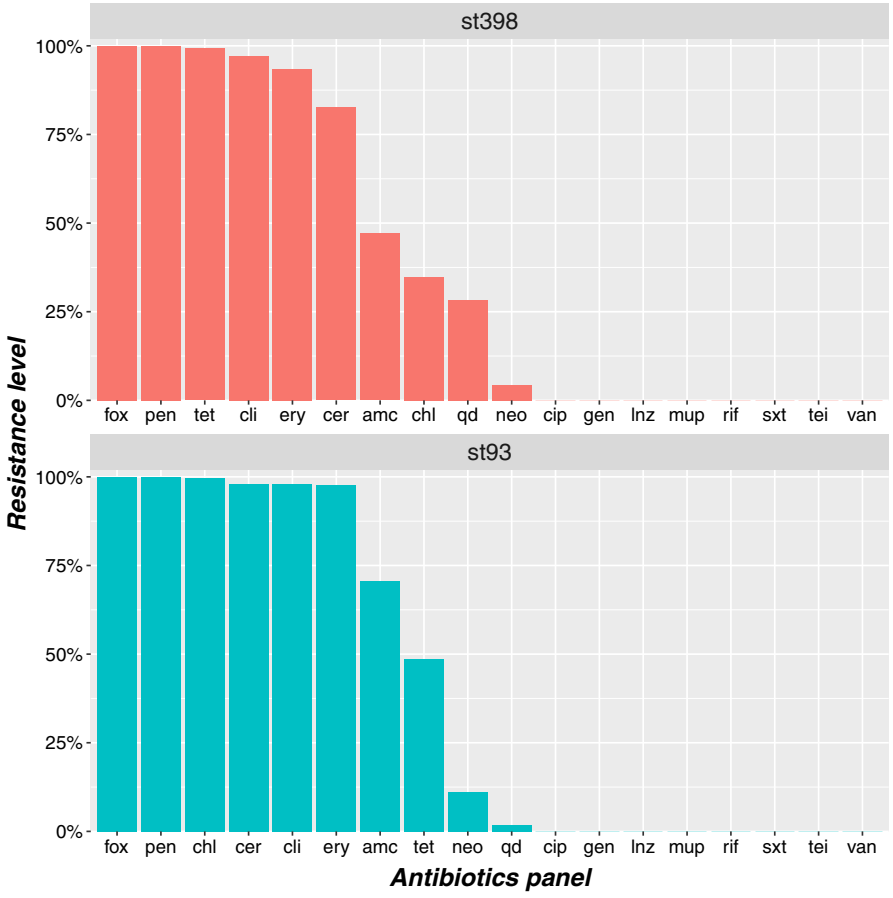

FIGURE 2 Proportion of phenotypic resistance detected by disc diffusion amongst all ST398 and ST93 isolates collected from pigs and environment on a piggery in Australia experiencing an MRSA outbreak in piggery workers.*amc (amoxicillin–clavulanate), cer (ceftiofur), chl (chloramphenicol), cip (ciprofloxacin), cli (clindamycin), ery (erythromycin), fox (cefoxitin), gen (gentamicin), lnz (linezolid), mup (mupirocin), neo (neomycin), pen (penicillin), qd (quinupristin–dalfopristin), rif (rifampicin), sxt (trimethoprim/sulfamethoxazole), tei (teicoplanin), tet (tetracycline) and van (vancomycin)

Australia (Coombs et al., 2016). ST93 has been reported to be the most virulent strain amongst CA-MRSA, including ST8 (USA300) (Chua et al., 2011). Although ST93 is a predominant CA-MRSA in Australia and recently has replaced dominant HA-MRSA strains in healthcare settings, this strain has never been isolated from pigs or pig environment until recently. This is the first time that ST93 was reported as a pig-adapted strain as well as potential occupational hazard amongst piggery workers on this study farm (Sahibzada et al., 2017; Sahibzada, Hernández-Jover, Jordan, Thomson, & Heller, 2018). A national survey is required to identify whether ST93 is present in other pig herds in Australia. One-third of the ST93 (31%) isolates in this study did not have PVL encoding genes. In previous reports involving isolates procured directly from human cases, up to 100% of ST93 were reported as PVL positive (Coombs et al., 2016; Harch et al., 2017). The presence of PVL, regarded as a virulence factor in human isolates, is used as a marker to distinguish CA-MRSA from other MRSA (HA- and LA-MRSA). The absence of PVL among a large proportion of ST93 in this scenario indicates a potential adaptation of this clone to the new porcine host and its environment—a phenomenon previously described by Sahibzada et al. (2017). It is possible that ST93 could further acquire (or re-acquire) genetic traits in the presence of selection pressure that would enhance its ability to colonize humans, pigs or both.

Our results show that dry sows are less likely to carry MRSA (42.2%) compared to the other stages of production, in the production system that we sampled. Broens et al. (2012) observed similar results when conducting a study at multiple farms in Germany, and a lower carriage in dry sows (33.3%) compared to farrowing sows (77.3%), weaners (79.6%), growers (86.6%) and finishers (69.6%) was observed. Similarly, Dorado-Garcia et al. (2015) found a significantly ($p < .001$) lower risk of MRSA carriage among dry sows compared to other age groups (weaners OR 10.4, farrowing OR 4.0, growers OR1.1 and finishers OR 4.2), using a multivariable model where samples were collected from diverse farms most likely under different managemental practices. In a previous study on this farm, an association between MRSA carriage in workers and pig age groups has also been established where working with dry sows was recognized as having decreased odds for MRSA carriage in piggery workers compared to other age groups (Sahibzada et al., 2018), which is in line with the low proportion of MRSA carriage found in dry sows in this study. However, other factors such as type of contact (direct and indirect), duration of exposure, the intensity of work, personal hygiene and personal health status also play an important role in MRSA carriage in workers. The association of MRSA carriage with age observed in this study and other reports could be due to a number of factors, including management. For example, dry sows are usually maintained at low stocking density with less antimicrobial exposure (Postma et al., 2016; van Rennings et al., 2015) compared to other pig age groups. In contrast, weaners and growers, potentially housed at a greater stocking density and with greater antimicrobial exposure (DANMAP, 2016; Sjölund et al., 2016), were found to have a high MRSA carriage in this study. Weaners are likely to carry MRSA from the suckling stage, acquired either from contaminated environment or from their MRSA-positive mother (horizontally as well as vertically) (Moodley, Latronico, & Guardabassi, 2011; Verhegghe et al., 2013) with a tendency to carry on to the next production stages (Weese, Zwambag, Rosendal, Reid-Smith, & Friendship, 2011). In addition, weaning is recognized as the most stressful event in a pig's life due to a series of demanding changes, including nutritional, social (maternal separation) and physiological factors, which can negatively impact pig health, growth and feed intake (Campbell, Crenshaw, &

TABLE 2 Multiple drug-resistance phenotypes of MRSA isolates (n = 490) collected in pigs and environment on a single pig enterprise in Australia experiencing an MRSA outbreak among piggery workers. The table shows resistance pattern for each strain, ST398 and ST93

Resistance pattern	ST398% (n)	ST93% (n)
bla, tet	2.9 (4)	0
bla, chl	0	1.14 (4)
bla, chl, tet	0	0.85 (3)
bla, chl, cli, tet	3.62 (5)	0.28 (1)
bla, chl, cli, ery	0.72 (1)	50.14 (176)
bla, cli, ery, tet	60.14 (83)	0.28 (1)
bla, chl, cli, ery, tet	1.45 (2)	35.04 (123)
bla, cli, ery, neo, tet	2.17 (3)	0
bla, chl, cli, ery, qd, tet	26.81 (37)	1.14 (4)
bla, chl, cli, ery, neo, tet	0.72 (1)	10.54 (37)
bla, chl, cli, ery, neo, qd, tet	1.45 (2)	0.57 (2)

Abbreviations: bla = cefoxitin, amoxicillin–clavulanate, ceftiofur, cefoxitin and penicillin, chl = chloramphenicol, cli = clindamycin, ery = erythromycin, neo = neomycin, qd = quinupristin–dalfopristin, tet = tetracycline

TABLE 3 Results of univariable analysis showing the risk (risk ratio) of detecting resistance to specific drugs in MRSA swabs from pigs (n = 465) exposed to an MRSA outbreak in piggery workers broken down by different pig age groups with dry sows as the comparison

	Amoxicillin–clavulanate	Tetracycline	Chloramphenicol
Pig age group	$p < .001$	$p < .001$	$p = .001$
Dry sows	1	1	1
Farrowing	0.9 (0.73–1.12)	0.66 (0.51–0.84)	0.98 (0.85–1.13)
Weaner	1.29 (1.04–1.6)	1.71 (1.38–2.13)	1.27 (1.09–1.47)
Grower	0.88 (0.7–1.1)	1.25 (1.01–1.57)	1.02 (0.88–1.18)
Finisher	1.03 (0.8–1.32)	1.08 (0.83–1.41)	1.09 (0.92–1.29)

Polo, 2013). These events that make pigs susceptible to bacterial infections and subsequently attract the most antimicrobial usage compared to any other stage (Sjölund et al., 2016). It has been established that levels of gram-positive organisms, including *S. aureus*, increase in the post-weaning period (Baele et al., 2001); however, less is known about specific CA- or LA-MRSA age-related patterns, particularly in Australian swine production facilities.

This study also found that weaners were more likely to carry ST93 in comparison with the other production age groups, on the farm that was studied. Although the reason for this association is not clear, it could be due to selection pressure exerted by the pattern of antimicrobials used in this particular cohort on this farm. For example, a study analysing the genomic and phenotypic traits of a highly prevalent hospital-associated (HA)-MRSA strain ST239 in Australia revealed that the usage of glycopeptides and daptomycin in hospitals readily favour the selection of this clone (Baines et al., 2015). The overall weaning stress and widespread use of antimicrobials in weaners could have contributed to the strain carriage and antimicrobial resistance in this stage of production, but there is no conclusive evidence to support this notion. The results of this study indicate the importance of MRSA strain prevalence in pigs, and a high carriage of a certain strain in pigs could reflect the carriage of in-contact humans.

Previously, host jumping events and acquisition of novel genetic elements have been demonstrated for ST5 (human-to-poultry host jump) (Lowder et al., 2009) and ST398 (human-to-pigs host jump) (Price et al., 2012). In line to this, we have found a lower than expected carriage of human-associated virulence factors, *lukF-PV* and *lukS-PV* and *sak/scn/chp*, among a large proportion of ST93 suggesting a new pig host adoption (Sahibzada et al., 2017). In the future, new strains such as CA-MRSA ST93 could adopt the pig as a new host which may represent the largest reservoir host and become challenging zoonotic agents. More work is needed towards a comprehensive understanding of the emergence and successful adaption of CA-MRSA in the pig environment.

No MRSA isolates in this study exhibited resistance/reduced susceptibilities to ciprofloxacin (a fluoroquinolone) or vancomycin (a glycopeptide), which are both classified as critically important for use in humans (WHO, 2017). Vancomycin is particularly important in the treatment of staphylococcal disease in humans because it is the last line of defence. A number of studies performed in Europe have found high percentages of resistance in pig MRSA isolates against fluoroquinolones (over 30%) (Crombé et al., 2012), aminoglycosides (up to 70%; Conceição et al., 2017) and streptogramins (up to 40%) (Boost, O'Donoghue, & Guardabassi, 2015). Australia has not only restricted the use of fluoroquinolone and glycopeptides in humans but also does not permit the use of these drugs in food-producing animals (Cheng et al., 2012). Avoparcin (a glycopeptide) was only used in chicken-meat production in Australia prior to 1999 at which stage it was withdrawn (APVMA, 2004). Imposing this strict control and regulation over the use of high importance antimicrobials in food animals is likely the reason for low resistance to these agents.

The current study identified a significant association between antimicrobial resistance and age group of the pig. MRSA isolates collected in weaners showed a significantly greater risk of resistance to chloramphenicol, amoxicillin–clavulanate and tetracycline when compared to other age groups. The use of amoxicillin and tetracycline are common in piggeries, and the use of these antimicrobials is not uncommon in weaners to prevent disease (Jordan et al., 2009; van Rennings et al., 2015). While chloramphenicol is not used in pigs in Australia, florfenicol, belonging to the same class of antibiotics, is still utilized and may confer cross-resistance to chloramphenicol. It is recognized that a high proportion of the overall antimicrobial use across all pig production stages occurs in weaners (DANMAP, 2016; Sjölund et al., 2016). Although antimicrobial usage has been associated with carriage of resistance in pigs, other management practices such as biosecurity and farm hygiene also play an important role in the prevalence and spread of MRSA in pigs (Alt et al., 2011; Dorado-Garcia et al., 2015).

The differences in MRSA carriage and antimicrobial resistance across different pig age groups highlights the importance of consideration of pig age groups while formulating a control strategy in pigs. It is suggested to avoid staff moving between working on different age groups of pigs within a single shift, where possible, to reduce the chances of MRSA spread between different ages, or when working in multiple sheds is required, changing clothing and use of shed specific boots is recommended where possible. Further investigation is required to study the risk factors such as antimicrobial usage, biosecurity practices and hygiene practices that prevail in Australian piggeries and are putatively able to be varied, that could potentially be associated with MRSA emergence, adaptation and continued circulation in pigs. And where possible, eradication measures may be implemented; however, eradication measures are costly. The issue of who pays for eradication of a resistant pathogen that poses a risk to humans, but is of no or minimal threat to the pigs on a farm, is of consequence in moving forward in the space of zoonotic AMR. It may be possible to include MRSA testing as part of an on-farm quality assurance programme where industry obtains a benefit. However, where the primary benefit of eradication or reduction is to humans it could be argued that a One-Health strategy with associated funding is required to ensure that limitations in response do not exist due to lack of financial incentive.

Although this study has identified associations between pig age groups raised in different sheds with MRSA carriage and certain antibiotics, no clustering within sheds was assessed in the regression models. This study has also shown that within a pig herd affected with a high prevalence of MRSA, and in the presence of a CA-MRSA strain, the resistance and virulence traits do not appear to be homogeneous across the population of isolates present. This has substantial ramifications for studies seeking to describe the epidemiology and ecology of CA-MRSA in pigs, given that a limited number of isolates clearly would not accurately characterize the epidemiologic scenario. Similarly, the need for epidemiologically sound sample size and well-designed study approach to understanding MRSA in pigs is also emphasized by the differences in sample prevalence amongst different age groups of pigs.

Using the modified RT-PCR assay (adopted in this study) in parallel to ST398 or only for non-ST398 isolates allows filtering of highly

prevalent ST93 and non-ST93 isolates and allows a high throughput performance at a low cost. Therefore, sequence-type classification for screening large numbers of MRSA isolates from piggeries targeting LA-MRSA ST398 and CA-MRSA ST93 can be an efficient and cost-effective strategy that could improve the logistical ease of large epidemiological studies without loss of sensitivity. The *spa* typing performed on selected MRSA isolates shows four different types. For ST93, only t202 was found, which is the predominant *spa* type hitherto associated with this clone in Australia (Coombs et al., 2012). In ST398, three different *spa* types (t011, t2510 and t8100) and an unknown type were found. All three types, t011, t2510 and t8100 are associated with ST398 in Europe (EFSA, 2009; Lozano et al., 2011), with t011 being one of the most frequently reported (Broens, Graat, van der Wolf, van De Giessen, & De Jong, 2011; Crombé et al., 2012). The *spa* type t011 found in this study is considered to have decreased affinity for carriage and infection in humans (Ballhausen et al., 2014). PCR-HRM curve analysis is also proved to be a robust, reliable and cost-effective test for *spa* typing and can be used as a tool to differentiate between CA-MRSA- and LA-MRSA-related *spa* types (Ghorashi, Heller, Zhang, & Sahibzada, 2020).

5 | CONCLUSION

A strong association between age class of pigs and MRSA carriage was found in this study: dry sows had the lowest carriage and weaners the highest. Age class should, therefore, be purposefully included in the design of surveillance and outbreak investigations involving pigs as should be the potential for concurrent existence of multiple MRSA sequence types within herds. Moreover, heterogeneity in the anti-biograms of MRSA detected in this outbreak demonstrates the need for adequate intensity of sampling of animals and isolates. Clone specific real-time PCR is a quick and cost-effective method for resolving the MRSA sequence-type status of livestock.

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CONFLICT OF INTEREST

None of the authors involved in this manuscript has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this report.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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