Antimicrobial resistance and genetic diversity of *Staphylococcus aureus* collected from livestock, poultry and humans

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ABSTRACT

Staphylococcus aureus is one of the most prominent nosocomial, community and farm acquired bacterial infections among animals and human populations. The main purpose of our study was to identify and characterize antimicrobial resistance (AMR) among *Staphylococcus aureus* isolated from livestock, poultry and humans and to further identify the associated genes. *Staphylococcus aureus* isolates from human, bovine, swine and poultry were collected from different laboratories across the United States collected between 2003 and 2016. Antimicrobial susceptibility testing for 13 antimicrobials was performed and conventional PCR was used to detect the presence of the nuc gene, mec gene, and to detect int1 gene. Associations between the presence of mec and intl and specific AMR profiles were determined. Antimicrobial resistance was detected in all four host categories, with the highest overall rates found in swine, 100% resistant to tetracycline, 88% to penicillin and 64% clindamycin. The next highest was found among humans with 81.6% of isolates resistant to penicillin followed by 44% to clindamycin and 43% to erythromycin. Among beef cattle isolates, 63.2% were resistant to penicillin, 15.8% resistant to clindamycin and 15.8% to erythromycin. No isolates from any of the hosts were resistant to linezolid. Among poultry isolates, the highest AMR was found to clindamycin, followed by erythromycin and penicillin. Among dairy cattle, highest resistance was found to penicillin, followed by chloramphenicol and gentamicin. Dairy cattle were the only host category with isolates that are resistant to trimethoprim-sulfamethoxazole. Of the 220 isolates detected by latex agglutination, 217 were confirmed to be *S. aureus* via PCR of the nuc gene, 21.4% were positive for the mecA gene. Swine had the highest prevalence of the mecA gene, followed by humans, poultry and beef cattle. This study has demonstrated a high occurrence of penicillin resistance among all *S. aureus* isolates. There were differences observed between host species with tetracycline resistance being the highest among swine isolates and clindamycin being highest in poultry isolates. No detection of oxacillin resistance was found in isolates from dairy cattle but was found in isolates from all of the other host species, 94% of which contained the mecA gene.

1. Introduction

Emerging and existing antimicrobial drug resistance (AMR) is a major public health concern with global relevance to overall animal health, specifically livestock. AMR is often only detected following treatment failure, during which time the potential to spread disease to susceptible species is high. Although the development and spread of AMR is multifactorial, it has been shown that AMR definitively increases the severity of foodborne illness [1,2] and other clinical infections in people [3–5] as well as animals.

Among all staphylococci, *S. aureus* is the most invasive species and an etiological agent of diverse human and animal maladies, including skin infections, abscesses, food poisoning, toxic shock syndrome, septicemia, endocarditis, and pneumonia [6–8]. Additionally, *S. aureus* is one of the most prominent causes of nosocomial- and community- acquired bacterial infections worldwide [9–11] enhancing the need to determine if the AMR of isolates from humans and other species is significant.

Penicillin was the first antibiotic mass produced for use in humans. Although initially it was highly effective for treatment of *S. aureus* infections, today over 90% of human *S. aureus* strains are resistant to this antibiotic. Antimicrobial resistance genes, including those genes encoding penicillin resistance, can be found on mobile genetic elements

such as plasmids, transposons, integrons [12,13]. Mobile genetic elements (MGEs) constitute only 25% of the staphylococcal genome [14], and they encode many putative virulence factors and antimicrobial resistance mechanisms. Thus, MGEs have an important role in bacterial adaptability and survival [15].

The main objective of our study was to phenotypically and genotypically identify AMR in *Staphylococcus aureus* isolates from livestock, poultry, livestock environment (swine and poultry) and humans. The findings from this study increases our understanding of host differences so surveillance for AMR bacteria in humans and food-producing animals could be improved.

As per the U.S. Department of Health and Human Services [16], investing in advanced diagnostics and increasing surveillance for antibiotic-resistant zoonotic and animal pathogens is needed, especially as a means to strengthen detection and control of antibiotic resistance. This study directly addresses the White House AMR initiative by classifying disease-causing *Staphylococcus aureus* infecting both humans and livestock according to their AMR patterns and molecular markers. The results of this study could be used to address dangerous AMR infections, including *S. aureus*, originating from livestock as well as from the human hospitals.

In the past decades, indiscriminate use and abuse of existing antibiotics has led to proliferation of antibiotic resistance in microbes [17] and consequently an increasing number of clinical failures in bacterial mediated diseases [18–20]. Several genetic carriers are responsible for the emergence and prevalence of AMR, such as plasmids and transposons. One way of detecting AMR is through detection and characterization of integrons. Integrons are genetic elements that acquire and incorporate gene cassettes that code for antibiotic resistance. Integrons are linked to mobile DNA elements (i.e. transposons and/or conjugative plasmids) and are horizontally transmissible [18,21–24]. Integrons associated with AMR have been detected in many bacterial species, including methicillin-resistant *S. aureus* (MRSA) [25]. In addition to MRSA, methicillin susceptible *S. aureus* (MSSA) have been found to contain integrons and can be resistant to at least 6 other antibiotics [26]. MSSA strains are capable of acquiring the methicillin resistance coding *mecA* gene cassette from MRSA [27].

There are five different classes of integrons, each encoding a distinct integrase gene [28], class 1 integrons being the most common type in clinical isolates of the Enterobacteriaceae [29]. The *S. aureus* strains that carried class 1 integrons was highest in those found to be multi-drug resistant *S. aureus* (MDR-SA) [30]. They were found in 72.6% of *S. aureus* clinical isolates [31].

In a study of *S. aureus* isolated from US meat and poultry samples, 96% were resistant to at least one antibiotic [32] and 78.4% of integrase-positive strains were multi-drug resistant. In the US, livestock-associated *S. aureus* can also be found in humans; likewise, human strains of *S. aureus* have also been found in US livestock [33]. Similar findings have been reported in the Netherlands [34]. This indicates a need to assess characteristics shared between *S. aureus* isolates derived from both humans and animals. Thus, understanding the host differences of AMR as well as AMR gene carriage will be an essential step to effectively manage pathogen transmission, especially within enclosed farm environments and hospitals and thus, reduce the number of new cases among humans, livestock, and poultry.

This work reveals an innovative way of improving detection of antibiotic-resistant zoonotic and animal pathogens nationwide. The diversity of hosts from which *S. aureus* AMR strains can be isolated suggests that much can be learned to improve our current approaches to mitigate the spread of multi-drug resistant pathogens.

2. Materials and methods

Ethical approval: Live animals were not used for the study.

2.1. Sample collection and culturing methods

Staphylococcus aureus isolates from human, bovine, swine and poultry hosts were collected from various repository laboratories across the United States representing time period between 2003 and 2017. Three-hundred and four isolates were initially collected from the laboratories located in Colorado, Pennsylvania, Washington, Ohio and Iowa. Only 254 isolates indicated appropriate host metadata. Of those, 220 isolates were identified as *S. aureus* by latex agglutination [35,36]. Single isolates were grown overnight in brain heart infusion broth at 35 °C. After overnight incubation, a 1 ml aliquot was removed, added to 1 ml of glycerol and frozen at −80 °C for further testing.

Molecular amplification of the *nuc, mecA* and *integron* cassette genes using multiplex PCR:

If an isolate was tested positive by latex agglutination, PCR of the *nuc* gene was used to confirm *S. aureus* [37]. PCRs were also conducted for the methicillin resistance gene (*mec*) [37] and for class 1 integron gene cassettes [22].

A portion of each *S. aureus* stock isolate was scraped into a separate microcentrifuge tube, thawed, and centrifuged for 5 min at 5000 x*g*. The supernatant was removed, and each pellet was resuspended in molecular grade water in a 1:3 ratio (10 μl cell pellet suspended in 30 μl water). A total of 5 μL of each washed, resuspended isolate was used as template and added to the following multiplex PCR master mix reaction to detect *nuc* gene. A total of 5 μL of each resuspended isolate was used as template in the following multiplex PCR mastermix for a 25 μl total reaction volume: 12.5 μl 2× Qiagen Multiplex PCR master mix (Qiagen, Valencia, CA), 2.0 μM primer mix (containing 2 μM of each primer: *nuc* forward primer sequence: 5′- AGC CAA GCC TTG ACG AAC TAA −3′; *nuc* reverse primer sequence: 5′- GCG ATT GAT GGT GAT ACG GTT - 3′; *mecA* forward primer sequence: 5′- GTA GAA ATG ACT GAA CGT CCG ATA A −3′; *mecA* reverse primer sequence: 5′- CCA ATT CCA CAT TGT TTC GGT CTA A - 3′; integron forward primer sequence: 5′- GGC ATC CAA GCA GCA AGC -3′; integron reverse primer sequence: 5′- AAG CAG ACT TGA CCT GAT - 3′), 2.5 μl Q-Solution (Qiagen, Valencia, CA), and 2.5 μl molecular grade water. Each 25 μl reaction was overlaid with 30 μl Chill Out wax (Bio-Rad, Hercules, CA) to prevent evaporation and placed into an MJ Research 60 place thermal cycler (Bio-Rad). Thermal cycling conditions consisted of an initial incubation at 95 °C for 10 min to activate the polymerase, followed by 35 cycles of 94 °C for 30 s, 56 °C for 2 min, 72 °C for 1.5 min, and a final extension incubation at 72 °C for 10 min. Following amplification, samples were run on a 1.5% agarose gel. Samples containing 279 bp products were recorded as *nuc* gene positive, samples containing 310 bp were recorded as *mecA* gene positive, and samples containing 1000, 1200, or 1600 bp amplicons were recorded as containing one or more class I integron gene cassette.

Two positive control samples for class I integrons with sizes of 1,000, 1,200, and 1,600 bp were included (5 pg total) with each PCR [22]. *Staphylococcus aureus* ATCC 25923 was included with each PCR as a positive control for the *nuc* gene product.

2.2. Antimicrobial susceptibility testing

To demonstrate and characterize antimicrobial resistance, antibiotic susceptibility testing (AST) was conducted on the 220 isolates by the Kirby Bauer (disk diffusion) method according to the Clinical Laboratory Standards Institute (CLSI) guidelines [38]. Isolates were plated on Mueller Hinton II agar. Antibiotics tested included ceftaroline (CPT-30), chloramphenicol (C-30), ciprofloxacin (CIP-5), clindamycin (CC-2), erythromycin (E-15), gentamicin (GM-10), linezolid (LZD-30), cefoxitin (FOX-30; surrogate for oxacillin per CLSI), penicillin G (P-10), rifampin (RA-5), tetracycline (TE-30) and sulfamethoxazole/ trimethoprim (SXT-23.75/1.25). Quality control was performed using a *S. aureus* positive control for beta-lactamase resistance (ATCC 29213), a *S. aureus* positive control for methicillin resistance (ATCC 43300), and a general *S. aureus* AST control for Mueller Hinton agar standardization (ATCC 25923).

Antimicrobial resistance patterns were deduced using NARMS (National Antimicrobial Resistance Monitoring System) panels and breakpoints selected according to CLSI recommendations.

In addition, reduced susceptibility to vancomycin was determined by plating three 10 μl drops of a standardized concentration of bacteria (0.5 McFarland), on brain heart infusion agar containing 6 μg/ml vancomycin and counting colonies. Disk approximation testing or D-zone reactions (erythromycin-inducible clindamycin resistance) were also recorded. This was conducted by placing an erythromycin and clindamycin disk 15 mm apart on an agar plate after isolate plating. All erythromycin resistant and clindamycin sensitive isolates were subjected to D-zone test using erythromycin (15 μg) and clindamycin (2 μg) according to Clinical and Laboratory Standards Institute [39]. If there was flattening of the zone of inhibition between the 2 disks and the zone resembles the letter "D," the test result was interpreted as positive for induction of clindamycin resistance. *Enterococcus faecalis* (ATCC 51299) was included as a positive control for vancomycin resistance. Reduced susceptibility to vancomycin was recorded if >1 colony per 10 μl drop was observed following 24 h incubation at 37 °C.

2.3. Statistical analysis

The data on AMR results of each antimicrobial were classified into susceptible, intermediate and resistant. The AMR data was considered as a binary variable (resistance or susceptible) for each drug tested. Those isolates which demonstrated an intermediate resistance to antibiotics were considered resistant for analyses. All data was visually represented using a heat map to depict the host variations on the patterns. The AST data on intermediate results was combined with resistance pattern to evaluate host differences using a logistic regression analysis for the comparisons with appropriate number of observations. All analyses were performed using SAS v9.4 (SAS Inc., Cary, NC).

3. Results

3.1. Antimicrobial resistance profiles of Staphylococcus aureus across host species

Seventy five percent of all *S. aureus* isolates were resistant to at least one antimicrobial drug and 40% were resistant to at least three antimicrobial drugs. The percentage of isolates found to be resistant to at least one antimicrobial drug among various hosts were: 68% of beef cattle, 42% of dairy cattle, 86% of human isolates, 64% of poultry, and 100% of swine isolates. Antimicrobial resistance was detected in all 5 host categories, with the highest overall rates found in swine, 100% resistance to tetracycline, 88% to penicillin and 64% clindamycin. The

next highest was found among humans with 81.6% of isolates resistant to penicillin followed by 44% to clindamycin and 43% to erythromycin. Among beef cattle isolates, 63.2% were resistant to penicillin, 15.8% resistant to clindamycin and 15.8% to erythromycin. None of the isolates from any of the hosts were resistant to linezolid. Among poultry isolates, the highest AMR was found to clindamycin in 40% of the isolates, 35.6% to erythromycin, and 33.3% to penicillin. Among dairy cattle, 39.4% of isolates were resistant to penicillin, followed by 9.1% resistance to chloramphenicol and 9.1% to gentamicin. Dairy cattle were the only host category with isolates that are resistant to trimethoprim-sulfamethoxazole.

The percentage of *S. aureus* isolates determined to be resistant to various antimicrobials via susceptibility testing are depicted by host category in Fig. 1. The isolates with intermediate resistance were combined with resistant isolates.

Oxacillin resistance was found among 22.7% of total *S. aureus* isolates, highest among swine (36%), followed by humans (31.6%), poultry (20%) and beef cattle (5.3%). None of the dairy cattle isolates were resistant to oxacillin.

3.2. mecA gene prevalence

Latex agglutination identified 220 isolates serologically as *S. aureus*. Fig. 2 depicted AMR patterns of *S. aureus* with characterization of genes by host category. Briefly, the isolates were distributed in the five host categories: beef cows ($n = 19$), dairy cows ($n = 33$), poultry ($n = 45$), swine ($n = 25$) and humans ($n = 98$). Of the 220 isolates, 217 were confirmed to be *S. aureus* via PCR of the *nuc* gene. The three isolates negative for the *nuc* gene but serologically positive for *S. aureus* were included in the study as described by Hoegh et al. (2014) where *nuc*-negative clinical isolates of *S. aureus* were described [40].

Of the 220 *S. aureus* isolates tested, 21.4% (47/220) were positive for the *mecA* gene. Among the host categories studied, swine had the highest prevalence of the *mecA* gene, with 32% (8/25) of the samples. Humans had the second highest prevalence at 31% (30/98), followed by poultry at 18% (8/45) and beef cattle at 5% (1/19). There was no detection of the *mecA* gene in the dairy cow isolates. There was one isolate each from poultry, swine and human that were resistant to cefoxitin (surrogate for oxacillin) and did not carry the *mecA* gene.

3.3. Integron detection

None of the *S. aureus* isolates contained class 1 integrons, hence no further analysis was performed.
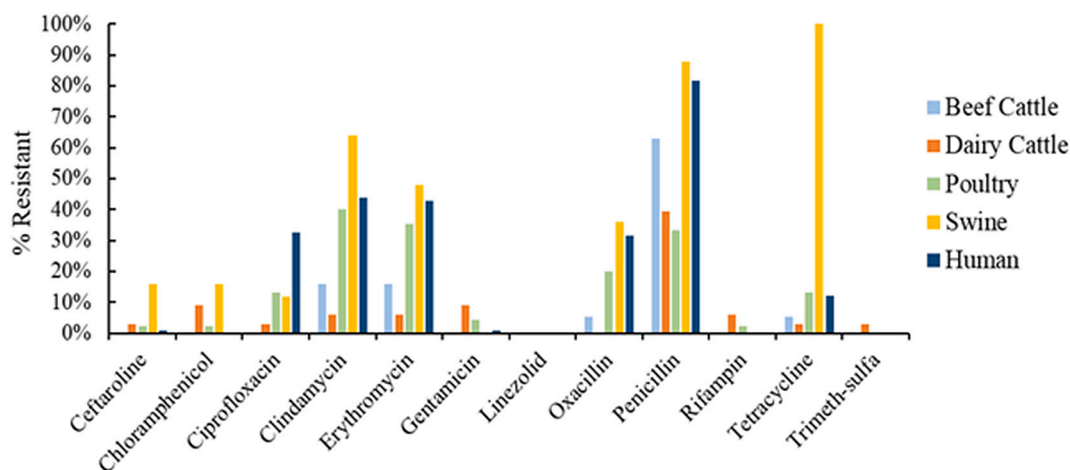

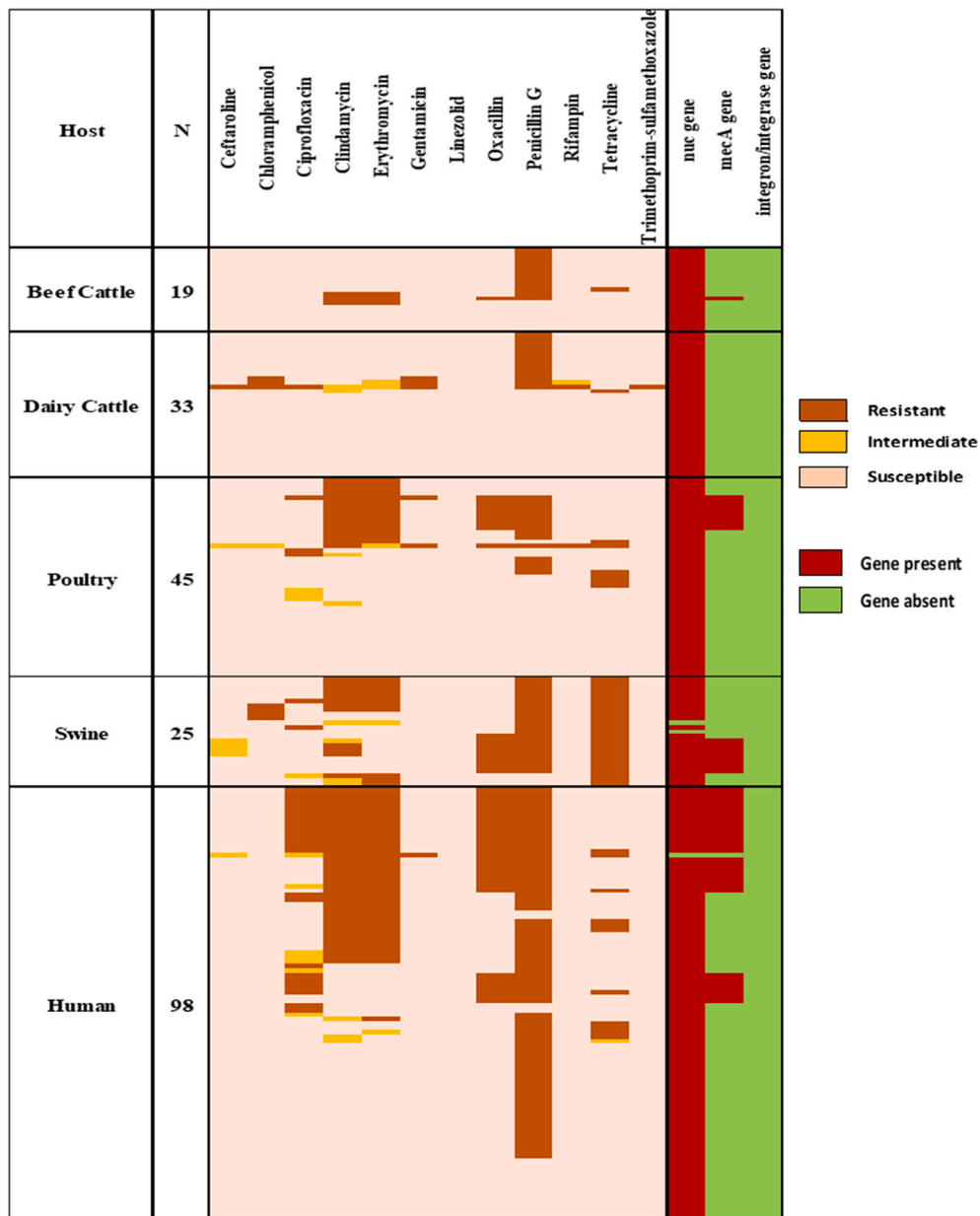**Fig. 1.** Antimicrobial resistance of *S. aureus* by host category.

Fig. 2. Antimicrobial resistance patterns of *S. aureus* with gene characterization by host category.

Table 1
Likelihood of clindamycin, erythromycin and penicillin resistance among host species.

Comparison of host species	Clindamycin				Erythromycin				Penicillin			
	OR	95% CI		P-value	OR	95% CI		P-value	OR	95% CI		P-value
Beef Cattle vs Dairy Cattle	2.91	0.44	19.20	0.27	2.91	0.44	19.20	0.27	2.64	0.82	8.45	0.10
Beef Cattle vs Poultry	0.28	0.07	1.11	0.07	0.34	0.09	1.35	0.12	3.43	1.12	10.50	0.031*
Swine vs Dairy Cattle	27.55	5.31	142.95	<0.001*	14.31	2.80	73.08	0.0014*	11.28	2.80	45.47	0.0007*
Swine vs Beef Cattle	9.48	2.16	41.61	0.0029*	4.92	1.14	21.23	0.033*	4.28	0.93	19.65	0.06
Swine vs Poultry	2.67	0.97	7.33	0.057	1.67	0.62	4.52	0.31	14.67	3.78	56.93	0.0001*
Swine vs Human	2.27	0.92	5.64	0.077	1.23	0.51	2.97	0.64	1.65	0.45	6.12	0.45
Poultry vs Dairy Cattle	10.33	2.20	48.63	0.0031*	8.55	1.81	40.47	0.0068*	0.77	0.30	1.96	0.58
Human vs Dairy Cattle	12.12	2.75	53.45	0.001*	11.62	2.63	51.30	0.0012*	6.84	2.88	16.25	<0.001*
Human vs Beef Cattle	4.17	1.14	15.24	0.031*	4.00	1.09	14.62	0.04*	2.59	0.90	7.51	0.08
Human vs Poultry	1.17	0.57	2.40	0.66	1.36	0.66	2.82	0.41	8.89	3.98	19.85	<0.001*

OR = Odds Ratio.

CI = Confidence Interval.

* Significant at p-value<0.05

3.4. Association of AMR profiles and host differences

The statistical analyses to find differences between host species was performed for penicillin, clindamycin and erythromycin as there were not adequate number of isolates for analyses that were resistant to the other antimicrobials (Table 1).

The isolates from swine had a significantly higher likelihood of penicillin, clindamycin and erythromycin resistance individually when compared to isolates from dairy cattle. Additionally, swine isolates were more likely to have clindamycin and erythromycin resistance when compared to isolates from beef cattle. There were no statistically significant differences detected in resistance to the three antimicrobials between dairy and beef cattle isolates.

Penicillin resistance was significantly higher in swine, beef cattle and human isolates when compared to poultry isolates. However, clindamycin and erythromycin resistance were significantly higher in poultry isolates when compared to dairy cattle isolates.

Human isolates were more likely to have clindamycin and erythromycin resistance when compared to dairy and beef cattle isolates, and significantly higher number of penicillin resistant isolates when compared to dairy cattle and poultry isolates.

4. Discussion

Antimicrobial resistance in bacterial infections continues to be a high-priority health concern in humans as well as animals. In humans, there is a high cost burden associated with treating infections caused by AMR bacteria due to longer hospitalization periods and high costs associated with complex treatment plans [41]. *Staphylococcus aureus* is prevalent in human and animal populations and is known to have developed resistance to different antimicrobials. Humans are the primary reservoir for *S. aureus* and serve as a hub for host switching events, allowing *S. aureus* to successfully infect multiple species [42–45]. Confirmed cases of interspecies transmission between pigs, humans and cattle, emphasize the importance of understanding host specific antimicrobial resistance patterns for *S. aureus* to study transmission to animal and public health [44,46]. This study aimed to identify the prevalence of MRSA isolates and the antimicrobial resistance profiles of *S. aureus* isolates collected from humans and major food animal groups.

In the current study, swine and human *S. aureus* isolates had the highest detection of the *mec*A gene, a finding that was also reported by previous studies [45,47–50]. This study suggests that MRSA was more likely to be isolated from swine populations when compared to poultry or cattle among *S. aureus* collected for laboratory analysis. The prevalence of methicillin resistance in swine isolates poses a potential risk for public health. In 2005, a swine-adapted lineage of MRSA was detected that could infect humans while having a low pathologic potential in swine [51]. Another study found a near 100% rate of nasal MRSA carriage in Dutch slaughter pigs, posing a potential risk for pig to human transmission [52].

The host groups with the lowest *mec*A detection in this study were dairy and beef cattle isolates, with no dairy cow isolates positive for the *mecA* gene. *S. aureus* is a common cause of contagious mastitis infections in dairy cows and can be difficult to control with antimicrobials alone. Hygiene protocols to prevent contagious mastitis transmission are used extensively in the dairy industry and could possibly explain the low rates in dairy cattle isolates [53–55]. This study's low detection of methicillin resistance in *Staphylococcus aureus* isolates is similar to a previous study reporting the prevalence in Canadian dairy cows at 0.5% in 2008 [56].

All 220 *S. aureus* isolates in this study were susceptible to linezolid, including MRSA isolates. Linezolid has been explored as a more effective treatment for MRSA when compared to vancomycin, but linezolid-resistance has been reported in MRSA isolates in human hospitals [57–59]. So far, the majority of linezolid-resistance has been observed in human isolates of *S. aureus*, but low rates of resistance in swine has been noted by other studies. Similar to this study, no resistance has been reported for cattle or chicken isolates [60–62].

This study found human, dairy cattle and beef cattle isolates to were more likely to be resistant to penicillin. Numerous studies have confirmed high rates of penicillin resistance in *S. aureus* isolates due to the development of the enzyme penicillinase/ β-lactamase causing inactivation of beta-lactam drugs [63–66]. When comparing dairy and beef cattle isolates, resistance profiles differ in that beef cattle isolates were resistant to erythromycin while no resistant dairy cattle isolates were detected. Dairy cattle isolates were the only isolates to show resistance to ceftaroline and trimethoprim-sulfamethoxazole. Trimethoprim-sulfamethoxazole resistance in dairy cattle isolates has been demonstrated to be found in milk products by Abdeen et al. (2020) [66] and low-level resistance to ceftaroline in the isolates from this study was consistent with results obtained by Abdel-Moein et al. (2019) [60].

One hundred percent of the swine isolates in this study were resistant to tetracyclines, which supports the findings of Dierikx et al. (2016) [52] and Conceição et al. (2017) [67]. This resistance to tetracycline is characteristic of swine isolates of *S. aureus* and can be linked back to a host-switching event from humans to pigs [44]. It could also be linked to the tetracyclines used as feed additives in swine operations. The tetracycline resistance gene *tet(M)* has been described to be universally prevalent among livestock-associated MRSA but absent from human-isolates. Tetracyclines are also used at higher amounts in farmed animals when compared against human medicine, which could explain the persistent detection of this resistance [46]. Resistance in swine isolates was detected against penicillin, clindamycin and erythromycin and supporting previous studies [52,67].

Antimicrobial resistance has been attributed to horizontal gene transfer of AMR genes via plasmids, transposons and integrons [68]. Integrons have been detected in *S. aureus* isolates from poultry litter, human hospitals, and bovine milk, however; this study detected no integrons in the 220 isolates, including MRSA isolates [69–71].

This study was performed on isolates collected from multiple states over the course of 14 years (2003–2017). The sample matrices were not included in the data that was received from any of the laboratories nor was the geographical locations of the sample origins. These limitations of non-availability of enough metadata have the possibility to impact the detection of integrons, as decreasing identification rates of integrons have been noted over time [70]. The isolates were mostly representative of the laboratory submission of clinical as well as surveillance samples.

From the data gathered in this study, it is clear that *S. aureus* isolates do not rely specifically on integrons for AMR gene transfer, as our study observed antimicrobial resistance in the absence of integron detection.

5. Conclusions

This study has demonstrated a high occurrence of penicillin resistance in *Staphylococcus aureus* isolates collected from all of the host species. There were differences observed between host species with tetracycline resistance being the highest among swine isolates and clindamycin being highest in poultry isolates. No detection of oxacillin resistance was found in isolates from dairy cattle but was found in isolates from all of the other host species. Ninety four percent of the *S. aureus* isolates that were resistant to oxacillin contained the *mecA* gene. Drug resistant *S. aureus* is medically significant because there is a narrow range of other treatment options in animals or humans.

CRediT authorship contribution statement

Sangeeta Rao: Funding acquisition, Conceptualization, Methodology, Data curation, Formal analysis, Supervision, Validation,

Visualization, Writing – original draft, Writing – review & editing. **Lyndsey Linke:** Funding acquisition, Conceptualization, Methodology, Data curation, Writing – review & editing. **Roberta Magnuson:** Funding acquisition, Conceptualization, Methodology, Data curation. **Linzy Jauch:** Validation, Visualization, Writing – original draft, Writing – review & editing. **Doreene R. Hyatt:** Funding acquisition, Conceptualization, Writing – review & editing.

Declaration of Competing Interest

None.

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