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J. Dairy Sci. 103:3431–3446 https://doi.org/10.3168/jds.2019-17141

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ABSTRACT

Staphylococcus aureus is recognized worldwide as one of the main contagious mastitis agents in cattle and can express a set of antimicrobial resistance genes and virulence-associated genes that explain the wide range of outcomes of intramammary infections. Staphylococcus aureus strains are heterogeneous: their different resistance and virulence patterns, associated with hostlevel factors and treatment factors, are related to the severity of infection. The aim of this study was to determine phenotypic antibiotic susceptibility, occurrence of selected antimicrobial resistance genes and other virulence genes in 93 S. aureus strains isolated from clinical mastitis in 6 countries: Argentina, Brazil, Germany, Italy, the United States (New York State), and South Africa. These isolates were tested against a total of 16 drugs (amoxicillin-clavulanate, ampicillin, cefazolin, cefoperazone, cefquinome, enrofloxacin, erythromycin, gentamicin, kanamycin, lincomycin, oxacillin, penicillin, rifampin, spiramycin, sulfamethoxazole/trimethoprim, tylosin) by minimum inhibitory concentration (MIC) assay, and examined for the presence of 6 antibiotic-resistance genes (blaZ, mecA, mecC, ermA, ermB, ermC) and 6 virulence-associated genes (scn, chp, sak, hla, hlb, sea) via PCR analysis. The phenotypic results of this study revealed the presence of 19.4% penicillin-resistant strains, whereas 22.6% of the strains were classified as having resistance (5.4%) or intermediate resistance (17.2%) to erythromycin. Most (96.8%) of the isolates were inhibited by cephalosporins, and all were susceptible to amoxicillin-clavulanate. Two strains (1 from Germany, 1 from Italy) were resistant to oxacillin and were positive for mecA. Among the other antimicrobial resistance genes, the most frequently detected was blaZ

(46.2%), and 32.3% of the isolates were positive for erm genes: ermC (21.5%) and ermB (10.8%). The most prevalent virulence gene was hla (100%), followed by hlb (84.9%) and sea (65.6%). These results show a low prevalence of antibiotic multidrug resistance in S. aureus isolates, even if the detection of selected antimicrobial resistance genes did not always correspond with the occurrence of phenotypic antibiotic resistance; the immune evasion cluster gene prevalence was quite low in the samples analyzed.

Key words: dairy cow, mastitis, Staphylococcus aureus, virulence gene, antimicrobial resistance gene, MIC

INTRODUCTION

Mastitis is a common disease of dairy cows and a major concern for the dairy industry because of economic losses due to decreased animal health and increased antibiotics usage (Heikkilä et al., 2018; Gussmann et al., 2019). Staphylococcus aureus is one of the major agents of contagious mastitis, responsible for mainly subclinical but also clinical infections in cattle worldwide (Barkema et al., 2006). This pathogen, in combination with both the bovine host and environmental factors, is characterized by low cure rates compared with other mastitis pathogens because of its capability to acquire antibiotic resistance and produce a wide array of virulence factors (Malinowski et al., 2002; Moroni et al., 2006; Sakwinska et al., 2011; Gao et al., 2012). Higher parity is associated with a lower probability of cure, which is lower also in older cows with high SCC and in cows infected in hindquarters during early and mid-lactation (Sol et al., 1997). Although S. aureus responds poorly to treatment with many different antimicrobial agents, antibiotic therapy still plays a significant role in the prevention and cure of bovine staphylococcal mastitis. The infection of cows with increasingly antibiotic-resistant strains can cause several therapeutic problems and is one of the main reasons for monitoring drug resistance (Pol and Ruegg, 2007; USDA, 2007; Saini et al., 2012a). The measurement of S. aureus antimicrobial resistance using phenotypic susceptibility tests, such as disk diffusion or MIC assay, is essential in order to select the most appropriate and efficient therapy (Walker, 2006). These methods can be combined with molecular analysis, as phenotypic S. aureus resistance to the most commonly used antimicrobials is related to the expression of antibiotic-resistance genes (Cockerill, 1999). The genes associated with resistance to β-lactams are often detected in S. aureus isolates from bovine milk samples, because β-lactams have been widely used to prevent and treat mastitis cases for several decades (Saini et al., 2012a; Saini et al., 2012b). Among the genes encoding β-lactamase, blaZ is responsible for resistance to penicillin (Olsen et al., 2006), whereas the mecA (Sawant et al., 2009) and mecC (Paterson et. al., 2014) genes confer resistance to methicillin, a semisynthetic penicillinase-resistant penicillin. Another concern is the emergence of erythromycin resistance regulated by the ermA, ermB, and ermC genes encoding different ribosomal methylases (Gatermann et al., 2007). In addition to antibiotic-resistance genes, S. aureus strains can harbor virulence genes in different combinations, thereby expressing factors used to attach, colonize, invade, and infect the host, which contribute largely to the establishment and severity of bovine mastitis (Jarraud et al., 2002). Many S. aureus virulence factors can be described as toxins (Otto, 2014). Among them, the hemolysins are cytolytic toxins able to lyse different types of cells. Staphylococcus aureus isolates from bovine mastitis, in particular, show a high level of expression of α-toxin (hla), exhibiting dermonecrotic and neurotoxic effects on a wide range of mammalian cells (Berube and Bubeck Wardenburg, 2013). β-Toxin (hlb) is a sphingomyelinase that damages cell membranes rich in this lipid; it is produced abundantly by isolates of animal origin (Clarke and Foster, 2006), as it increases the adherence of S. aureus to bovine mammary epithelial cells (Magro et al., 2017). Staphylococcus aureus can also produce a wide array of enterotoxins (from SEA to SEQ): SEA, in particular, is able to promote the ability of S. aureus to evade host immune defenses by negatively affecting the activity of

neutrophils (Xu et al., 2014). The gene for enterotoxin A (sea) belongs to the immune evasion cluster (IEC), which also includes scn, chp, sak, and other enterotoxin genes (sep, sek, or seq; Cuny et al., 2015). This cluster can interfere with host immunity and is common in methicillin-resistant S. aureus (MRSA) strains isolated from humans (McCarthy and Lindsay, 2013); in animals, it was previously detected in MRSA isolates from horses (Cuny et al., 2015). Because S. aureus virulence and antimicrobial resistance profiles are associated with specific genotypes (Fournier et al., 2008), a greater understanding of the epidemiology of S. aureus genotypes in dairy herds may help monitor the emergence of antimicrobial-resistant strains associated with their virulence characteristics. The aim of this study was to determine (1) the phenotypic antimicrobial susceptibility and (2) the prevalence of selected antimicrobial resistance genes and other virulence genes in 93 S. aureus isolates from clinical mastitis milk samples collected in 6 different countries; all these isolates were previously genotyped by RS-PCR (Monistero et al., 2018).

MATERIALS AND METHODS

Herd and Isolate Enrollment Criteria

Ninety-three S. aureus isolates from single-quarter (Q) and composite (C) milk samples of cows with clinical mastitis were collected between 2012 and 2017 from 76 farms in 6 countries: Argentina, Brazil, Germany, Italy, the United States (New York State), and South Africa (Table 1). Farms enrolled in the present and prior study (Monistero et al., 2018) were required to have a minimum of 120 lactating cows, to participate in monthly DHI testing or to use monthly California Mastitis Test for all lactating animals, to use a milking routine including fore-stripping of quarters for detection of mastitis, and to have a farm survey once a year by sending quarter or composite milk samples to the reference laboratory. The isolates were selected based on a non-probability convenience sample, and only isolates from clinical mastitis were selected for this study. Considering a within-herd mastitis prevalence of 20%, of which 10 to 15% were clinical cases, this yielded 2 to 4 isolates per farm.

Sample Collection

Milk samples were collected by farm personnel trained to detect mastitis cases. After disinfection of teat ends and discarding the first streams of foremilk, milk was collected in 10-mL sterile vials, labeled with cow number and quarter. Clinical mastitis was defined as visibly abnormal milk from a mammary quarter (Wenz et al., 2001; Ruegg, 2011). Milk samples were stored at 4°C and shipped to their respective laboratories. Ten microliters of each sample were plated on blood agar plates, and bacterial cultures were evaluated after 24 h of aerobic incubation at 37°C. Staphylococcus aureus colonies were round, smooth, substantial, opaque, characterized by hemolysis, and positive in the tube coagulase test (Cookson, 1997). One colony of each S. aureus isolate was subcultured and stored at −20°C. The isolates were shipped frozen on either dry ice or wet ice, depending on the distance, to the Department of Veterinary Medicine (University of Milan, Italy). Upon arrival, isolates were stored at −20°C.

Antimicrobial Susceptibility

Testing The MIC of 16 antimicrobials were determined for 93 S. aureus isolates, using the broth dilution test according to the procedure described in Clinical and Laboratory Standards Institute (CLSI) guidelines VET01-A4 (CLSI, 2013). The MIC were performed with a customized commercial microdilution MIC system (Micronaut-S MIC Mastitis, Merlin Diagnostika, GmbH, Bornheim, Germany) used for routine laboratory testing of mastitis isolates. Results were interpreted using

available CLSI resistance breakpoints according to VET01-S2 guidelines (CLSI, 2013) or other breakpoints reported in the literature if CLSI standards were not established. If breakpoints were differentiated for host species, cattle breakpoints were selected. The CLSI breakpoints were used for the following antimicrobials: amoxicillin-clavulanate, ampicillin, cefazolin, enrofloxacin, erythromycin, gentamicin, oxacillin, penicillin, rifampin, and sulfamethoxazole/trimethoprim. The breakpoints standardized by Société française de microbiologie (2018) were used for lincomycin, kanamycin, and spiramycin; literature references were used for cefoperazone (Feßler et al., 2012), cefquinome (Lang et al., 2002), and tylosin (Simjee et al, 2011). Furthermore, the MIC inhibiting the growth of 90% of the isolates (MIC90) was calculated for each antimicrobial. The MIC plates reading was performed manually, and the last concentration of antimicrobial that did not show turbidity or a deposit of cells at the bottom of the well was recorded. The MIC value of each isolate, expressed as micrograms per milliliter, was defined as the lowest concentration of the antimicrobial agent that completely inhibited the growth after the incubation period. Staphylococcus aureus ATCC 29213 was used as a quality-control strain in each MIC batch, and a double negative control was used for each plate. The antimicrobials used on the plate were selected based on their activity against mastitis pathogens and on their registrations for dairy cattle. Ceftiofur was not included in the plate, because this drug is not approved for mastitis treatment in Europe, as opposed to the United States and Canada. Considering that third-generation cephalosporins are generally not advised for S. aureus treatment, specific testing for this antimicrobial was not performed in the present study

β-Lactamase Detection

Phenotypic β-lactamase activity was determined using the nitrocefin-based test (nitrocefin disks, SigmaAldrich, St. Louis, MO), performed according to the manufacturer's instructions and to VET08 guidelines (CLSI, 2018). Staphylococcus aureus ATCC 29213 and S. aureus ATCC 25923 were used as positive and negative controls, respectively.

Molecular Analysis DNA Extraction.
DNA was extracted from the isolates following the protocol described by Cremonesi and coworkers (2006). After the measurement of its amount and quality using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), DNA was stored at −20°C.

Molecular Characterization.
The DNA extracted from the 93 isolates was amplified via specific PCR analysis to determine the occurrence of 6 antibiotic-resistance genes (blaZ, mecA, mecC, ermA, ermB, ermC), the hemolysins (hla and hlb) and the IEC genes (chp, sak, scn, and sea). All these genes were investigated using primers and protocols described in literature (Table 2). Each PCR reaction contained a total of 12.5 µL of Phusion High-Fidelity Master Mix 2× (Thermo Fisher Scientific, Waltham, MA) for detection of blaZ, ermA, and hla or 12.5 µL of PCR Master Mix 2× (Thermo Fisher Scientific) to investigate the other genes considered; 0.2 µL of each primer (100 µM) were added to 2 µL of genomic DNA (5 ng/µL). As positive controls, S. aureus reference strains (ATCC 19040, ATCC 19041, ATCC 19048, ATCC 700699, or S. aureus isolates from the collection of IZSLER, previously analyzed by molecular tests) were used in each PCR assay (Table 3). All amplified PCR fragments were visualized on 2% agarose gel electrophoresis (GellyPhor, Euroclone, Milan, Italy), stained with ethidium bromide (0.05 mg/mL; Sigma-Aldrich), and visualized under UV transilluminator (BioView Ltd., Nes Ziona, Israel). A 100-bp DNA ladder (Finnzymes, Espoo, Finland) was included in each gel.

RESULTS

Antimicrobial Profiling and Virulence Profiling The 93 S. aureus strains analyzed in this study were identified with the same identification (ID) numbers used in the previous study (Monistero et al., 2018). All of them were positive for the gene for α-hemolysis (hla) but negative for a gene involved in host cell invasion (chp) and 2 antimicrobial resistance genes, 1 responsible for resistance to methicillin (mecC) and 1 conferring resistance to erythromycin (ermA). The MIC assay demonstrated 100% phenotypical susceptibility to tylosin and amoxicillin-clavulanate. Argentina. All of the 16 isolates from Argentina showed phenotypic intermediate or complete resistance to spiramycin, except 1 (ID 5); 7 (43.8%) isolates were not inhibited by erythromycin, and 5 (31.3%) were also not sensitive to lincomycin. Only 1 isolate (ID 12) was phenotypically resistant to ampicillin and penicillin. The nitrocefin-based method detected 2 (12.5%) β-lactamase-positive isolates (Table 4). The molecular analysis revealed that all strains carried ermC, except 1 (ID 2), which was negative for this gene. The ermC gene was the only erythromycinresistance gene found in Argentina, although blaZ was detected with a frequency of 18.8% (Table 4). The hlb gene was detected in 93.8% of the Argentinian strains. Among the IEC genes, the most prevalent was sea (56.3%), whereas sak was carried by 5 (31.3%) isolates, and none harboured scn (Table 4). Brazil. All 15 isolates collected in Brazil showed phenotypic intermediate or complete resistance to spiramycin, except 1 (ID 29). Among the Brazilian isolates, 46.7% were not susceptible to lincomycin, and 13.3% were not inhibited by the range of concentration tested for erythromycin. Out of the 15 isolates analyzed, 3 (20%) were found to be β-lactamase-positive by the nitrocefin-based method, also demonstrating in vitro resistance to ampicillin and penicillin, and 3 (20%) showed resistance to the combination of trimethoprim and sulfamethoxazole (Table 5). The genotypic results showed that 46.7% of the Brazilian strains were positive for blaZ, and all were negative for both mecA and erm genes (Table 5). As reported in Table 5, the gene for β-hemolysin (hlb) was present in 100% of the Brazilian strains. The majority (53.3%) of them carried sea, but none harbored the other IEC genes investigated, scn and sak. Germany. In the MIC assay, 94.1% of German isolates were phenotypically resistant to spiramycin. Out of 17 strains analyzed, 6 (35.3%) showed in vitro resistance to erythromycin and 5 (29.4%) to lincomycin. One isolate (ID 53) was also resistant to ampicillin, oxacillin, penicillin, and the 3 cephalosporins tested (cefazolin, cefoperazone, and cefquinome); this isolate was the only one detected by the nitrocefin-based method. Another single German isolate (5.9%) was classified as having intermediate resistance to cefoperazone, and another 2 (11.8%) were classified as resistant to sulfamethoxazole/trimethoprim (Table 6). As shown in Table 6, 47.1% of the German strains were potentially resistant to penicillin, harboring blaZ, whereas the erm genes were less prevalent, with 35.3% of the strains positive for ermB but none for ermC. The single strain phenotypically resistant to 9 different antimicrobials, including methicillin, was the only one (6.9%) positive for mecA. Most (64.7%) of the strains isolated from Germany were positive for hlb. The result related to the presence of the IEC genes indicated that 15 (88.2%) strains carried sea, but none possessed the virulence factors associated with suppressing innate immunity (scn and sak; Table 6). Italy. The results of antimicrobial susceptibility testing (Table 7) showed that more than a half (58.8%) of the isolates collected in Italy were not inhibited by spiramycin or penicillin or both. Out of 10 penicillin-resistant isolates, 9 (52.9%) also showed resistance to ampicillin, and 9 revealed phenotypic β-lactamase activity. One other isolate was detected by the nitrocefin-based method, for a total of 10 (58.8%) β-lactamase-positive isolates with this test. Besides penicillin and ampicillin, 1 isolate (ID 77) was not susceptible to the other 5 drugs (spiramycin, cefoperazone, enrofloxacin, oxacillin, and lincomycin). Two (11.8%) Italian isolates were considered resistant to gentamycin, and 1 (5.9%) also showed in vitro resis tance to kanamycin. Only 1 isolate (ID 79) was classified as having intermediate resistance to erythromycin. Of 17 Italian strains, 14 (82.4%) were potentially penicillin-resistant, carrying blaZ, and 5 strains (29.4%) also harbored ermC. A single strain (5.9%) was positive for mecA: this was phenotypically resistant to 7 different antimicrobials, including methicillin, but negative for the erm genes (Table 7). Table 7 shows that 94.1% of the strains isolated from Italy carried hlb; the sea gene was detected in 58.8% of the strains, but only 1 (ID 78)

was also positive for both scn and sak genes. United States (New York State). All American isolates, with 1 exception (ID 97), exhibited resistance to spiramycin. From these spiramycin-resistant isolates, 1 (ID 90) was classified as having intermediate resistance to erythromycin, 1 (ID 82) as having intermediate resistance to rifampicin, and 1 (ID 88) as resistant to lincomycin (Table 8). Among the antimicrobial resistance genes investigated, blaZ had a prevalence of 41.2%, and erm genes were not identified (Table 8). By contrast, the hlb gene was found to be quite diffused (88.2%); sea was carried by 52.9%, and scn and sak were not found (Table 8). South Africa. Among South African isolates, the highest rate of intermediate or complete resistance was found for spiramycin (100%), followed by erythromycin (36.4%). Of 11 isolates analyzed, 3 (27.3%) were phenotypically resistant to penicillin and ampicillin, but only 1 (ID 103) showed phenotypic β-lactamase activity; a second isolate (ID 108) was detected by the nitrocefinbased method, for a total of 2 (18.2%) β-lactamasepositive isolates. Only 1 isolate (ID 100) was classified as having intermediate resistance to lincomycin (Table 9). Of the 6 antimicrobial-resistance genes tested, blaZ and ermB were detected in the African strains with the same frequency (36.4%; Table 9). Of 11 South African strains analyzed, results (Table 9) showed that 7 (63.6%) carried the gene for β-hemolysin (hlb). Among the IEC genes, sak and sea were detected in 100% and 90.9% of the strains, respectively; 1 (ID 103) of them was also positive for scn.

Association Between Phenotypic Resistance and Resistance Genes

The phenotypic results showed that most (93.6%) of the isolates had intermediate resistance or complete resistance to at least 1 of the 16 antimicrobial agents tested. Analyzing the resistance to multiple classes of antimicrobials, 57.0% of isolates were resistant or intermediate to 1 class of antimicrobials, 25.8% to 2 different classes, 8.6% to 3 different classes, and 2.2% (the 2 MRSA isolates) to more than 3. Table 10 reports all the raw MIC values and the MIC90 of the isolates for each antimicrobial tested. The MIC90 of all antimicrobials tested was lower than the resistance breakpoint, except for penicillin, ampicillin, spyramicin, and tylosin. The MIC assay (Table 10) revealed that 50 (53.8%) isolates were not inhibited by the range of concentrations tested for spiramycin, which was the antimicrobial with the highest rate of resistance. Of 93 isolates, 21 (22.6%) were classified as having intermediate resistance or resistance to erythromycin, 20 (21.5%) to lincomycin, 18 (19.4%) to penicillin, and 17 (18.3%) to ampicillin. The nitrocefin-based method detected a total of 18 (19.4%) isolates producing β-lactamase: 15 of these were also phenotypically resistant to penicillin, and 3 showed susceptibility to this drug. In estimating the occurrence of genes responsible for antibiotic resistance using PCR analysis, blaZ had the highest frequency (46.2%). Additionally, we investigated the presence of erm genes and mec genes that can confer resistance to erythromycin and methicillin, respectively. The genotypic results (Figure 1) showed that the resistance rate to erythromycin was 32.3%, and the most frequently detected erythromycin-resistance gene was ermC (21.5%), followed by ermB (10.8%). The prevalence of methicillin-resistant S. aureus strains was low among the isolates analyzed, as only 2 (2.2%) strains harbored mecA.

Figure 2 shows the association between the occurrence of genes conferring antibiotic resistance (x-axis) and laboratory-tested phenotypic resistance to antimicrobials (y-axis). The association was calculated as the sum of co-occurrences of genetic and phenotypic resistance to antibiotics, normalized over sample size (Buzydlowski, 2015). The molecular detection of the antibiotic-resistance genes was not always directly proportional to the phenotypic expression of these genes (Figure 2). The gene responsible for resistance to penicillin (blaZ) was the most prevalent (46.2%), but only 19.4% of the strains analyzed demonstrated phenotypic resistance to this drug; the same percentage (19.4%) of isolates were reported to be resistant due to a positive nitrocefin test result. All isolates that demonstrated phenotypic resistance to penicillin or β-lactamase activity carried the blaZ gene. In addition, 32.3% of the isolates were positive for erm genes, but the phenotypic results showed that 21 (22.6%) of the 93 strains analyzed were resistant (5.4%) or had intermediate resistance (17.2%) to

erythromycin; 10 (10.8%) of the intermediate erythromycin-resistant strains were negative for ermB or ermC. The 2 phenotypically oxacillin-resistant strains were the only ones that harbored mecA. Figure 3 shows the comparison of penicillin MIC distribution and frequency of blaZ-positive and blaZnegative isolates. The distribution of blaZ-positive MIC is scattered along the dilution range, without bimodal distribution and with most of the isolates (23) having the lower MIC. The blaZ-negative isolates are gathered in the last 2 MIC dilutions with a clear unimodal distribution. Figure 3 also displays the comparison of erythromycin MIC distribution and frequency of ermpositive and erm-negative isolates. In this case, ermpositive isolates show a bimodal distribution, having as cutoff the resistance breakpoint, whereas erm-negative isolates are distributed only behind the resistance cutoff, with the major frequency at 0.5 µg/mL.

DISCUSSION

Of the 93 S. aureus strains analyzed, only 2 European isolates harbored mecA. None were positive for mecC, a recently identified mecA homolog detected in humans and in a wide range of domestic and wild animals from different European countries (Schlotter et al., 2014). Our results confirmed the low prevalence of MRSA among S. aureus strains collected from bovine mastitis samples (Hendriksen et al., 2008; Silva et al., 2013; da Costa Krewer et al., 2015; Luini et al., 2015). The β-lactam antibiotics have been largely used to treat S. aureus mastitis for several decades, but their efficiency is reduced by bacterial β-lactamases. The blaZ gene, which encodes β-lactamase and confers resistance to penicillin (Olsen et al., 2006), was the most frequently detected resistance gene, found in 43 strains. Of these, 21, including the 2 MRSA isolates, were reported to be phenotypically resistant to penicillin based on MIC or nitrocefin-based test results. Therefore, the remaining 50% of the blaZ-positive isolates were phenotypically susceptible to penicillin, in agreement with previous results reported by Ruegg and collaborators (2015). Haveri et al. (2005) suggested that the occurrence of isolates with phenotypic resistance to a certain antibiotic might not always be proportional to the presence of the corresponding resistance gene. Considering the isolates susceptible to penicillin but positive for blaZ as potentially resistant (Haveri et al., 2005), our results confirmed that resistance to penicillin was the most frequently observed resistance mechanism, although with a lower prevalence than the over 60% reported by Malinowski and collaborators (2002, 2008). On the other hand, looking at the comparison of penicillin MIC distribution and the frequency of blaZ-positive and blaZ-negative isolates, most of the isolates had the lowest MIC. This discrepancy between phenotypic and genotypic results may demonstrate that the detection of genes does not necessarily implicate their expression; indeed, the percentage of isolates phenotypically resistant to penicillin was in agreement with previous results (Ruegg et al., 2015), reporting that the resistance rate to this drug has declined (Makovec and Ruegg, 2003), even with differences among geographical areas. Previous studies demonstrated that the occurrence of phenotypically penicillin-resistant S. aureus strains was higher in Argentina (40%; Gentilini et al., 2000) than in Germany (17%; Tenhagen et al., 2006) or in the United States (10%; Anderson et al., 2006). Accordingly, we found higher resistance rates in Argentina and Germany compared with New York State, but the rates were overall lower (12.5% in Argentina, 5.9% in Germany, and 0% in New York State). Of 18 phenotypically penicillin-resistant isolates, 17 were not inhibited even by the highest concentration of ampicillin tested, in accordance with previous studies (El Behiry et al., 2012; Jagielski et al., 2014). This outcome could be explained by the presence of blaZ in all these strains, because penicillin, as well as ampicillin, is inactivated by the β-lactamase encoded by blaZ. Among them, 3 were negative for the β-lactamase test; this discrepancy could be due to the lower sensitivity of the nitrocefin test compared with MIC assay and PCR analysis for the blaZ gene (Ferreira et al., 2017). All 93 isolates analyzed in this study were susceptible to the association of amoxicillin and clavulanate, with a very low MIC90 (0.5 µg/mL). Considering the uncertainties connected to laboratory methods for detection of β-lactamase-producing S. aureus strains, the use of amoxicillin and clavulanate could be

recommended when only phenotypic methods are available to test resistance to penicillin, given also its demonstrated efficiency in mastitis therapy (Güler et al., 2005). In addition to the genes responsible for resistance to β-lactams, we investigated the presence of the genes encoding resistance to erythromycin. We detected erm genes in 30 of the 93 isolates analyzed and found that ermC was the most prevalent gene, similar to the results of Aarestrup and Schwarz (2006) and Sawant et al. (2009). The phenotypic results showed that the MRSA strain collected in Germany was positive for ermB and was also classified as phenotypically erythromycin-resistant, whereas the other MRSA isolate from Italy was negative for both erm genes tested and was susceptible to erythromycin. Of 30 isolates positive for erm genes, 5 were classified as having complete resistance and 6 as having intermediate resistance to erythromycin. The susceptibility of the remaining 19 isolates could be due to lack of expression of methylases encoded by erm genes, in agreement with previous studies (Fluit et al., 2001). The other 10 isolates negative for erm genes showed intermediate resistance to erythromycin, but they were very close to the CLSI breakpoints; therefore, we cannot discount the possibility that some of them could be considered susceptible. Surprisingly, among the macrolides tested, we found a high number of isolates resistant to spiramycin but susceptible to erythromycin, when the genes responsible for resistance to erythromycin usually also confer resistance to other macrolides. The possibility of an uncorrected resistance breakpoint should be taken into account, and the MIC distribution could be helpful to analyze the data. Spiramycin MIC results (Table 10) show a bimodal distribution, with an epidemiological cutoff at 4 µg/100 mL, which seems to split the isolates into 2 different phenotypical populations and which corresponds to the resistance breakpoint. A greater number of isolates and dilution points in the area of resistance would be needed to assess the accuracy of the breakpoint. Therefore, possible bias due to an incorrect resistance breakpoint cannot be excluded, even if the epidemiological cutoff is consistent with the breakpoint for this set of data. Another possible explanation of this phenomenon could be the presence among the isolates of other genes encoding resistance to macrolides that have not been tested in this study. The emergence of macrolide-resistance genes conferring resistance to spiramycin but not to erythromycin has been described in Streptococcus uberis (Achard et al., 2008). The cephalosporins, usually classed into different generations based on their antimicrobial spectrum, are often used to treat mastitis in dairy ruminants (Moroni et al., 2005). Globally, there are intramammary formulations of first-generation cephalosporins (cefazolin, cephalexin, cephalotin, cephalonium, and cephapirin), second-generation (cefuroxime), third-generation (cephoperazone and ceftiofur), and fourth-generation (cefquinome; Moroni et al., 2005; Oliveira and Ruegg, 2014; Ruegg et al., 2015). We tested cefazolin as a first-generation cephalosporin, and cephoperazone and cefquinome as third- and fourth-generations, respectively. These last 2 antimicrobials, classified by the World Health Organization (WHO, 2019) as highestpriority critically important antibiotics (HPCIA), were tested in this study because they were included in the MIC plates used for routine testing. It is important to highlight that the use of third- and fourth-generation cephalosporins should be limited only to gram-negative bacteria that show resistance to antibiotics different from HPCIA (WHO, 2017). The use of other drugs, such as first-generation cephalosporins or amoxicillinclavulanate, should be preferred to these cephalosporins for the treatment of staphylococcal mastitis. Results showed that the 2 MRSA isolates both displayed resistance to cefoperazone (MIC 8 µg/mL). Moreover, the MRSA strain isolated in Germany had a MIC of 4 µg/mL, classified as intermediate, to cefazolin and cefquinome, whereas that isolated in Italy had a MIC of 2 µg/mL, classified as susceptible, to both drugs. The 2 MRSA isolates were resistant to oxacillin with a MIC value >4 µg/mL, outside the dilution range. These data highlight that only oxacillin or cefoxitin should be used to phenotypically assess the presence of MRSA, confirming the detection of the mecA gene, as advised by CLSI (2013). However, bovine mastitis caused by S. aureus strains positive for the mecA gene and treated by administration of cephalosporins show clinical outcomes with low probability of cure (Pol and Ruegg, 2007; Oliveira and Ruegg. 2014; Krömker and Leimbach, 2017). For this reason, antibiotic treatment decisions should be based not only on the diagnosis of the mastitis causative agents

obtained through microbiological and sensitivity test results but also on the identification of animals with high healing prospects (Krömker and Leimbach, 2017). The MIC90 was lower than the resistance breakpoint for the majority of the antimicrobials tested. This outcome was expected, in agreement with previous studies (Gentilini et al., 2000; Ruegg et al., 2015). Therefore, for many antimicrobials, we selected a dilution range wider in the lower side and narrow in the upper part, to possibly detect the presence of bimodal distribution also in the susceptibility dilution range. This reduced the possibility of investigating the extent of the resistance level for some antimicrobials, such as lincomycin, spiramycin, and tylosin. Analysis of the virulence profiles of the 93 S. aureus strains revealed that the 2 adhesion factors Hla and Hlb, also involved in host invasion (Moroni et al., 2011), were the most frequently detected. In fact, the genes for α- and β-hemolysins were found to be widely distributed in all the 6 countries analyzed, in agreement with Aarestrup et al. (1999). All strains were positive for hla (100%), and 79 (84.9%) also carried hlb. The gene for α-toxin is present in essentially all S. aureus strains (Monecke et al., 2014), including strains isolated from humans, whereas the β-toxin gene, whose activity may be important in the pathogenesis of mastitis, is more frequent in bovine rather than human isolates (Larsen et al., 2002). We also investigated the occurrence of the IEC genes that play an important role in human medicine (Baptistão et al., 2016), especially in infections caused by MRSA (McCarthy and Lindsay, 2013). This cluster comprises the staphylococcal complement inhibitor gene (scn) and the chemotaxis inhibitory protein (chp), which are located on an 8-kb region at the conserved 3′ end of β-hemolysin (hlb)-converting bacteriophages (βC-φs). The region at the conserved 3′ end encodes the genes sak, sea, or sep (van Wamel et al., 2006). Seven different IEC types (A to G) were previously identified, based on the occurrence of sea, sep, sak, chp, and scn genes, and type B (sak-chp-scn) was the most prevalent (van Wamel et al., 2006). The presence of this cluster in large animals was previously investigated in MRSA and methicillin-sensitive S. aureus (MSSA) isolates from different kinds of infections in pigs and horses as well as in humans with occupational exposure to pigs and horses (Cuny et al., 2015). Cuny and collaborators (2015) detected the IEC genes only in MRSA from horse clinics and the colonization of veterinary personal, probably for a re-adaptation to humans. Acquisition of the IEC is probably one of the first steps in the process of adaptation to animals and including loss or acquisition of genetic elements (Schijffelen et al., 2010). In this context, we investigated the occurrence of the IEC genes in S. aureus isolates from bovine intramammary infection. Our results showed that neither strain of MRSA found in our study carried these genes: the one from Germany was negative for all of them, whereas that from Italy carried only the gene encoding for enterotoxin A. However, the IEC type D was detected in 1 Italian and 1 South African MSSA, carrying scn, sak, and sea. This cluster type has been described as quite common in human MRSA (van Wamel et al., 2006). The gene chp was overall absent, but sak was present in 31.3% of Argentinian and 100% of South African strains. Our findings are in accordance with a recent paper (Magro et al., 2017) reporting that only bovine isolates were devoid of such prophage, probably because the untruncated hlb is necessary in ungulates for the different structure of erythrocyte membranes. In contrast to our results, the IEC genes were reported to be quite frequent in a recent Tunisian study on S. aureus strains collected from cow and ewe milk: IEC type B was predominant (Khemiri et al., 2019). The sea gene was carried, on average, by half of the isolates from each country, with the exception of Germany and South Africa, where the prevalence of this gene was 88.2% and 90.1%, respectively.

CONCLUSIONS

Although it is not straightforward to generalize to the global dairy population, given the limited size and the non-probability convenience sampling scheme of this study, our results strengthen the knowledge of the virulence and antibiotic-resistance patterns of S. aureus strains in dairy cows. Few specific genes were frequently detected in the strains analyzed, suggesting that they could be related to the ability of S. aureus to colonize the host. The blaZ gene was identified in most of the isolates

analyzed, even though the detection of this gene, as well as of erm genes, did not correspond with the relative occurrence of phenotypic resistance; further research will be necessary to validate phenotypic susceptibility testing and genotypic testing. Notwithstanding the ongoing alert on methicillin-resistant S. aureus strains, only 2 MRSA isolates were identified in this study; all other isolates were susceptible to oxacillin, and the majority were also susceptible to most antimicrobials tested. Therefore, the presence of highly multidrug-resistant isolates was low, and the emergence of widespread S. aureus multidrug resistance is limited to MRSA, in agreement with the previously mentioned works. The results of the present work show that the prevalence of antimicrobial-resistant S. aureus strains vary depending on country and herd, but collection of more comprehensive data through collaboration with a greater number of countries can provide further information on the spreading of antibiotic resistance; these findings could be used for further studies or metaanalysis on combined data sets. To date, the results suggest that it is necessary to maintain the described antimicrobial resistance trends, making antibiotic treatment decisions based on rapid diagnostic and resistance tests, and to keep an adequate level of surveillance on the presence of MRSA in dairy cattle, to avoid the spreading of these strains in dairy cattle populations and beyond.

ACKNOWLEDGMENTS

The authors acknowledge Belinda Gross and Janie C. Lee (Animal Health Diagnostic Center, Cornell University, Ithaca, NY) for their valuable revision of the English text. The authors have not stated any conflicts of interest.

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