Different distribution of antimicrobial resistance genes and virulence profiles of Staphylococcus aureus strains isolated from clinical mastitis in six countries

Journal:	*Journal of Dairy Science*
Manuscript ID	JDS.2019-17141.R2
Article Type:	Research
Date Submitted by the Author:	18-Nov-2019

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Key Words:	dairy cow, mastitis, Staphylococcus aureus, antimicrobial resistance

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1 **Interpretive summary:**

2 Different distribution of antimicrobial resistance genes and virulence profiles of *Staphylococcus* 3 *aureus* strains isolated from clinical mastitis in six countries. *Staphylococcus aureus* is frequently 4 isolated from bovine mastitis and responds poorly to treatment with different antibiotics, although 5 antimicrobial therapy continues to play an important role in the prevention and cure of staphylococcal 6 mastitis. Despite the evolution of increasingly antimicrobial-resistant *S. aureus* strains, we classified 7 most of the isolates analysed as susceptible to most of the antimicrobials tested. To conclude, our 8 study shows a low prevalence of antibiotic multidrug resistance in *S. aureus* isolates.

9

10 **Running head: Differential *S. aureus* antibiotic-resistance**

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12 **Different distribution of antimicrobial resistance genes and virulence profiles of**
13 ***Staphylococcus aureus* strains isolated from clinical mastitis in six countries**

14

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48 those of the writer and may not in any circumstances be regarded as stating an official position
49 of the European Commission.**

50

51 ## ABSTRACT

52 *Staphylococcus aureus* is recognized worldwide as one of the main contagious mastitis agents in
53 cattle and can express a set of antimicrobial resistance genes and virulence associated genes that
54 explain the wide range of outcomes of intramammary infections. *S. aureus* strains are heterogeneous:
55 their different resistance and virulence patterns, associated with the host-level factors and treatment
56 factors, are related to the severity of infection. The aim of this study was to determine phenotypic
57 antibiotic susceptibility, occurrence of selected antimicrobial resistance genes and other virulence
58 genes in 93 *S. aureus* strains isolated from clinical mastitis in 6 different countries (Argentina, Brazil,
59 Germany, Italy, U.S. (New York State) and South Africa). These isolates were tested against a total
60 of 16 drugs (amoxicillin/clavulanate, ampicillin, cefazolin, cefoperazone, cefquinome, enrofloxacin,
61 erythromycin, gentamicin, kanamycin, lincomycin, oxacillin, penicillin, rifampin, spiramycin,
62 trimethoprim/sulfamethoxazole, tylosin) by MIC assay, examined for the presence of 6 antibiotic
63 resistance genes (*blaZ, mecA, mecC, ermA, ermB, ermC*) and 6 virulence associated genes (*scn*, *chp*,
64 *sak*, *hla, hlb, sea*) by PCR analysis. The phenotypic results of this study revealed the presence of
65 19.4% penicillin-resistant strains, while 22.6% of the strains were classified as having resistance
66 (5.4%) or intermediate resistance (17.2%) to erythromycin. Most (96.8%) of the isolates were
67 inhibited by cephalosporins and all of them were susceptible to amoxicillin/clavulanate. Two strains
68 (1 from Germany, 1 from Italy) were resistant to oxacillin and were positive for *mecA*. Among the
69 other antimicrobial-resistance genes, the most frequently detected was *blaZ* (46.2%), while 32.3% of
70 the isolates were positive for *erm* genes: *ermC* (21.5%) and *ermB* (10.8%). The most prevalent
71 virulence gene was *hla* (100%), followed by *hlb* (84.9%) and *sea* (65.6%). These results show a low
72 prevalence of antibiotic multidrug resistance in S. aureus isolates, even if the detection of selected
73 antimicrobial resistance genes did not always correspond with the occurrence of phenotypic antibiotic
74 resistance, while the IEC gene prevalence was quite low in the samples analyzed.

75 **Key words:** dairy cow, mastitis, *S. aureus,* virulence gene, antimicrobial resistance gene, MIC

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76 ## INTRODUCTION

77 Mastitis is a common disease of dairy cows and a major concern for the dairy industry because of
78 economic losses due to the decreased animal health and increased antibiotics usage (Heikkilä et al.,
79 2018; Gussmann et al., 2019). *Staphylococcus aureus* is one of the major agents of contagious
80 mastitis, responsible for mainly subclinical but also clinical infections in cattle worldwide (Barkema
81 et al., 2006). This pathogen, in combination with both the bovine host and environmental factors, is
82 characterized by low cure rates compared to other mastitis pathogens because of its capability to
83 acquire antibiotic resistance and produce a wide array of virulence factors (Malinowski et al., 2002;
84 Moroni et al., 2006; Sakwinska et al., 2011; Gao et al., 2012). Higher parity is associated with a lower
85 probability of cure, that is lower also in older cows with high Somatic Cell Count (SCC) and in cows
86 infected in hindquarters during early and mid-lactation (Sol et al. 1997). Although this pathogen
87 responds poorly to treatment with many different antimicrobial agents, antibiotic therapy still plays a
88 significant role in prevention and cure of bovine staphylococcal mastitis. The infection of cows with
89 increasingly antibiotic-resistant strains can cause several therapeutic problems and is one of the main
90 reasons for monitoring drug resistance (Pol and Ruegg, 2007; USDA, 2007; Saini et al., 2012a). The
91 measurement of *S. aureus* antimicrobial resistance using phenotypic susceptibility tests, such as disk
92 diffusion or MIC assay, is essential to select the most appropriate and efficient therapy (Walker,
93 2006). These methods can be combined with molecular analysis, as phenotypic *S. aureus* resistance
94 to the most commonly used antimicrobials is related to the expression of antibiotic resistance genes
95 (Cockerill, 1999). The genes associated with resistance to β-lactams are often detected in *S. aureus*
96 isolates from bovine milk samples, because β-lactams have been widely used to prevent and treat
97 mastitis cases for several decades (Saini et al., 2012a; Saini et al., 2012b). Among the genes encoding
98 β-lactamase, *blaZ* is responsible for resistance to penicillin (Olsen et al., 2006), while *mecA* (Sawant
99 et al., 2009) and *mecC* (Paterson et. al., 2014) genes confer resistance to methicillin, a semisynthetic
100 penicillinase-resistant penicillin. Another concern is the emergence of erythromycin resistance
101 regulated by the *ermA, ermB* and *ermC* genes encoding different ribosomal methylases (Gatermann

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102 et al., 2007). In addition to antibiotic resistance genes, *S. aureus* strains can harbour virulence genes
103 in different combinations, thereby expressing factors used to attach, colonize, invade and infect the
104 host, which contribute largely to the establishment and severity of bovine mastitis (Jarraud et al.,
105 2002). Many *S. aureus* virulence factors can be described as toxins (Otto, 2014). Among them, the
106 haemolysins are cytolytic toxins able to lyse different types of cells. *S. aureus* isolates from bovine
107 mastitis, in particular, show a high level of expression of α-toxin (*hla*), exhibiting dermonecrotic and
108 neurotoxic effects on a wide range of mammalian cells (Berube et al., 2013). β-toxin (*hlb)* is a
109 sphingomyelinase that damages cell-membranes rich in this lipid; it is produced abundantly by
110 isolates of animal origin (Clarke et al., 2006) as it increases the adherence of *S. aureus* to bovine
111 mammary epithelial cells (Magro et al., 2017). *S. aureus* can also produce a wide array of enterotoxins
112 (from SEA to SEQ): SEA, in particular, is able to promote the ability of *S. aureus* to evade the host
113 immune defences by negatively affecting the activity of neutrophils (Xu et al., 2014). The gene for
114 enterotoxin A (*sea*) belongs to the immune evasion cluster (IEC) which also includes *scn, chp, sak*
115 and other enterotoxin genes (*sep*, *sek* or *seq*) (Cuny et al., 2015). This cluster can interfere with the
116 host immunity and is common in methicillin-resistant *S. aureus* (MRSA) strains isolated from humans
117 (McCarthy et al., 2013); in animals, it was previously detected in MRSA isolates from horses (Cuny
118 et al., 2015).

119 As *S. aureus* virulence and antimicrobial resistance profiles are associated with specific genotypes
120 (Fournier et al., 2008), a greater understanding of the epidemiology of *S. aureus* genotypes in dairy
121 herds may help to monitor the emergence of antimicrobial resistant strains associated with their
122 virulence characteristics.

123 The aim of this study was to determine i) the phenotypic antimicrobial susceptibility, and ii) the
124 prevalence of selected antimicrobial resistance genes and other virulence genes in the 93 *S. aureus*
125 isolates from clinical mastitis milk samples collected in 6 different countries; all these isolates had
126 been previously genotyped by RS-PCR (Monistero et al., 2018).

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127 **MATERIALS AND METHODS**

128 ***Herd and isolated Enrollment Criteria.***

129 Ninety-three *S. aureus* isolates from single-quarter (Q) and composite (C) milk samples of cows with
130 clinical mastitis were collected between 2012 to 2017 from 76 farms in six countries: Argentina,
131 Brazil, Germany, Italy, U.S. (New York State) and South Africa (Table 1). Farms enrolled in the
132 present and prior study (Monistero et al., 2018) were required to have a minimum of 120 lactating
133 cows, to participate in monthly DHI (Dairy Herd Improvement) testing or to use monthly CMT
134 (California Mastitis Test) for all lactating animals, to use a milking routine including fore-stripping
135 of quarters for detection of mastitis, and to have a farm survey once a year by sending quarter or
136 composite milk samples to the reference laboratory. The isolates were selected on the base of a non-
137 probability convenience sample and only isolates from clinical mastitis were selected for this study.
138 Considering a within-herd mastitis prevalence of 20%, of which 10-15% were clinical cases, this gave
139 2-4 isolates per farm.

140 ***Sample collection***

141 Milk samples were collected by farm personnel trained to detect mastitis cases. After disinfection of
142 teat ends and discarding the first streams of fore-milk, milk was collected in 10 mL sterile vials,
143 labeled with cow number and quarter. Clinical mastitis was defined as visibly abnormal milk from a
144 mammary quarter (Wenz el al., 2001; Ruegg, 2011).

145 Milk samples were stored at 4°C and shipped to their respective laboratories. Ten µl of each sample
146 were plated on blood agar plates and bacterial cultures were evaluated after 24 h of aerobic incubation
147 at 37°C. *S. aureus* colonies were round, smooth, substantial, opaque, characterized by hemolysis and
148 were positive in the tube coagulase test (Cookson, 1997). One colony of each *S. aureus* isolate was
149 sub cultured and stored at -20°C.

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150 The isolates were shipped frozen on either dry ice or wet ice, depending on the distance, to the
151 Department of Veterinary Medicine (University of Milan). Upon arrival, isolates were stored at -
152 20°C.

153 ***Antimicrobial Susceptibility Testing***

154 The Minimum Inhibitory Concentrations (MIC) of 16 antimicrobials were determined for 93 *S.*
155 *aureus* isolates using the broth dilution test according to the procedure described in Clinical and
156 Laboratory Standards Institute (CLSI) guidelines VET01-A4 (CLSI, 2013). MIC were performed by
157 a customized commercial microdilution MIC system (Micronaut-S MIC Mastitis, Merlin
158 Diagnostika, GmbH, Bornheim, DE) used for routine laboratory testing of mastitis isolates. Results
159 were interpreted using available Clinical and Laboratory Standard Institute (CLSI) resistance
160 breakpoints according to VET01-S2 guidelines (CLSI, 2013) or other breakpoints reported in the
161 literature if CLSI standards were not established. If breakpoints were differentiated for host species,
162 cattle breakpoints were selected. CLSI breakpoints were used for the following antimicrobials:
163 amoxicillin/clavulanate, ampicillin, cefazolin, enrofloxacin, erythromycin, gentamicin, oxacillin,
164 penicillin, rifampin and trimethoprim/sulfamethoxazole. The breakpoints standardized by the
165 "Société Française de Microbiologie (2018) were used for lincomycin, kanamycin and spiramycin,
166 literature references were used for cefoperazone (Feβler et al., 2012), cefquinome (Lang et al., 2002)
167 and tylosin (Simjee et al, 2011). Furthermore, the MIC inhibiting the growth of 90% of the isolates
168 (MIC_{90}) was calculated for each antimicrobial. The MIC plates reading was performed manually and
169 the last concentration of antimicrobial that did not show turbidity or a deposit of cells at the bottom
170 of the well was recorded. The MIC value of each isolate, expressed as µg/mL, was defined as the
171 lowest concentration of the antimicrobial agent that completely inhibited the growth after the
172 incubation period. *S. aureus* ATCC 29213 was used as a quality control strain in each MIC batch and
173 a double negative control was used for each plate. The antimicrobials used on the plate were selected
174 based on their activity against mastitis pathogens and on their registrations for dairy cattle. Ceftiofur

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175 was not included in the plate because this drug is not approved for mastitis treatment in Europe as
176 opposed to USA and Canada. Considering that third-generation cephalosporins are generally not
177 advised for *S. aureus* treatment, specific testing for this antimicrobial was not performed in the present
178 study.

179 ***β-Lactamase detection***

180 Phenotypic β-lactamase activity was determined using the nitrocefin-based test (Nitrocefin disks,
181 Sigma Aldrich) performed according to the manufacturer's instructions and to VET08 guidelines
182 (CLSI, 2018). *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 were used as positive and negative
183 control, respectively.

184 ***Molecular Analysis***

185 ***DNA Extraction.*** DNA was extracted from the isolates following the protocol described by
186 Cremonesi and co-workers (2006). After the measurement of its amount and quality by a NanoDrop
187 ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA), DNA was stored at
188 -20°C.

189 ***Molecular Characterization.*** The DNA extracted from the 93 isolates was amplified by specific PCR
190 analysis to determine the occurrence of 6 antibiotic resistance genes (*blaZ, mecA, mecC, ermA, ermB,*
191 *ermC*), the haemolysins (*hla* and *hlb*) and the IEC genes (*chp*, *sak, scn* and *sea*). All these genes were
192 investigated using primers and protocols described in literature (Table 2). Each PCR reaction
193 contained a total of 12.5 µl of Phusion High-Fidelity Master Mix 2x (ThermoFisher Scientific,
194 Waltham, MA, USA) for detection of *blaZ, ermA* and *hla* or 12.5 µl of PCR Master Mix 2x (Thermo
195 Scientific™, Waltham, MA USA) to investigate the other genes considered; 0.2 µl of each primer
196 (100 µM) were added to 2 µl of genomic DNA (5 ng/µl).

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197 As positive controls, *S. aureus* reference strains (ATCC 19040, ATCC 19041, ATCC 19048, ATCC
198 700699, or *S. aureus* isolates from the collection of IZSLER, previously analysed by molecular tests)
199 were used in each PCR assay (Table 3).
200 All amplified PCR fragments were visualized on 2% agarose gel electrophoresis (GellyPhor,
201 Euroclone, Milan, Italy), stained with ethidium bromide (0.05 mg/mL; Sigma Aldrich, Milan, Italy),
202 and visualized under UV transilluminator (BioView Ltd., Nes Ziona, Israel). A 100 bp DNA ladder
203 (Finnzymes, Espoo, Finland) was included in each gel.

204 **RESULTS**

205 ***Antimicrobial Profiling and Virulence Profiling***

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206 The ninety-three *S. aureus* strains analysed in this study were identified with the same identification
207 (ID) numbers used in the previous study (Monistero et al., 2019). All of them were positive for the
208 gene for α-haemolysis (*hla*), but negative for a gene involved in host cell invasion (*chp*) and two
209 antimicrobial resistance genes, one responsible for resistance to methicillin (*mecC*) and one of those
210 conferring resistance to erythromycin (*ermA*). The MIC assay demonstrated 100% phenotypical
211 susceptibility to tylosin and amoxicillin/clavulanate.

212 ***Argentina.*** All of the 16 isolates from Argentina showed phenotypic intermediate or complete
213 resistance to spiramycin, except one (ID 5); 7 (43.8%) isolates were not inhibited by erythromycin
214 and 5 (31.3%) were also not sensitive to lincomycin. Only one isolate (ID 12) was phenotypically
215 resistant to ampicillin and penicillin. The nitrocefin-based method detected 2 (12.5%) β-lactamase
216 positive isolates (Table 4).

217 The molecular analysis revealed that all the strains carried *ermC*, except one (ID 2) which was
218 negative for this gene; the *ermC* was the only erythromycin resistance gene found in Argentina, while
219 *blaZ* was detected with a frequency of 18.8% (Table 4).

220 The *hlb* gene was detected in 93.8% of the Argentinian strains. Among the IEC genes, the most
221 prevalent was *sea* (56.3%), while *sak* was carried by 5 (31.3%) isolates and none harboured *scn* (Table
222 4).

223 ***Brazil.*** All 15 isolates collected in Brazil showed phenotypic intermediate or complete resistance to
224 spiramycin, except one (ID 29). 46.7 % were not susceptible to lincomycin, while 13.3% were not
225 inhibited by the range of concentration tested for erythromycin. Out of 15 isolates analysed, 3 (20%)
226 were β-lactamase positive by the nitrocefin-based method, demonstrating also *in vitro* resistance to
227 ampicillin and penicillin, while 3 (20%) showed resistance to the combination of trimethoprim and
228 sulfamethoxazole (Table 5).

229 The genotypic results showed that 46.7% of the Brazilian strains were positive for *blaZ* and were all
230 negative for both *mecA* and *erm* genes (Table 5).

231 As reported in Table 5, the gene for β-haemolysin (*hlb*) was present in 100% of the Brazilian strains.
232 The majority (53.3%) of them carried *sea*, but none harboured the other IEC genes investigated (*scn*
233 and *sak*).

234 ***Germany.*** In the MIC assay, 94.1% of German isolates were phenotypically resistant to spiramycin.
235 Out of 17 strains analysed, 6 (35.3%) showed *in vitro* resistance to erythromycin and 5 (29.4%) to
236 lincomycin. One isolate (ID 53) was also resistant to ampicillin, oxacillin, penicillin and the 3
237 cephalosporins tested (cefazolin, cefoperazone and cefquinome); this isolate was the only one
238 detected by the nitrocefin-based method. Another single German isolate (5.9%) was classified as
239 intermediate resistant to cefoperazone and another 2 (11.8%) as resistant to
240 sulfamethoxazole/trimethoprim (Table 6).

241 As shown in Table 6, 47.1% of the German strains were potentially resistant to penicillin, harbouring
242 *blaZ,* while the *erm* genes were less prevalent with 35.3% of the strains positive for *ermB,* but none

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243 for *ermC*. The single strain phenotypically resistant to 9 different antimicrobials, including
244 methicillin, was the only one (6.9%) positive for *mecA*.

245 Most (64.7%) of the strains isolated from Germany were positive for *hlb*. The result related to the
246 presence of the IEC genes indicated that 15 (88.2%) strains carried *sea* but none possessed the
247 virulence factors associated with suppressing innate immunity (*scn* and *sak*) (Table 6).

248 ***Italy.*** The results of antimicrobial susceptibility testing (Table 7) showed that more than a
249 half (58.8%) of the isolates collected in Italy were not inhibited by spiramycin or penicillin or both.
250 Out of 10 penicillin-resistant isolates, 9 (52.9%) showed also resistance to ampicillin and 9 revealed
251 phenotypic β-lactamase activity. One other isolate was detected by the nitrocefin-based method, for
252 a total of 10 (58.8%) β-lactamase positive isolates with this test.

253 Besides penicillin and ampicillin, one isolate (ID 77) was not susceptible to the other 5 drugs
254 (spiramycin, cefoperazone, enrofloxacin, oxacillin and lincomycin). Two (11.8%) Italian isolates
255 were considered resistant to gentamycin and 1 (5.9%) of them showed *in vitro* resistance also to
256 kanamycin. Only one isolate (ID 79) was classified as intermediate resistant to erythromycin.

257 Out of 17 Italian strains, 14 (82.4%) were potentially penicillin-resistant, carrying *blaZ*, and 5 strains
258 (29.4%) also harbored *ermC*. A single strain (5.9%) was positive for *mecA*: this was phenotypically
259 resistant to 7 different antimicrobials, including methicillin, but negative for the *erm* genes (Table 7).

260 Table 7 shows that 94.1% of the strains isolated from Italy carried *hlb*; the *sea* gene was detected in
261 58.8% of the strains but only one (ID 78) was also positive for both *scn* and *sak* genes.

262 ***U.S. (New York State).*** All American isolates with one exception (ID 97) exhibited resistance to
263 spiramycin. From these spiramycin-resistant isolates, one (ID 90) was classified as intermediate
264 resistant to erythromycin, one (ID 82) as intermediate resistant to rifampicin, and one (ID 88) as
265 resistant to lincomycin (Table 8).

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266 Among the antimicrobial resistance genes investigated, *blaZ* had a prevalence of 41.2%, while *erm*
267 genes were not identified (Table 8).

268 On the contrary, the *hlb* gene was found to be quite diffused (88.2%); *sea* was carried by 52.9%,
269 while *scn* and *sak* were not found (Table 8).

270 ***South Africa.*** Among South African isolates, the highest rate of intermediate or complete
271 resistance was found for spiramycin (100%), followed by erythromycin (36.4%). Out of 11 isolates
272 analysed, 3 (27.3%) were phenotypically resistant to penicillin and ampicillin, but only one (ID 103)
273 showed phenotypic β-lactamase activity; a second isolate (ID 108) was detected by the nitrocefin-
274 based method, for a total of 2 (18.2%) β-lactamase positive isolates. Only one isolate (ID 100) was
275 classified as intermediate resistant to lincomycin (Table 9).

276 Of the 6 antimicrobial resistance genes tested, *blaZ* and *ermB* were detected in the African strains
277 with the same frequency (36.4%) (Table 9).

278 Out of 11 South African strains analyzed, the results (Table 9) showed that 7 (63.6%) carried the gene
279 for β-haemolysin (*hlb*). Among the IEC genes, *sak* and *sea* were detected in 100% and 90.9% of the
280 strains, respectively; one (ID 103) of them was also positive for *scn*.

281 ***Association between Phenotypic Resistance and Resistance Genes***

282 The phenotypic results showed that most (93.6%) of the isolates had intermediate resistance or
283 complete resistance to at least one of the 16 antimicrobial agents tested. Analysing the resistance to
284 multiple class of antimicrobials, 57.0% of isolates were resistant or intermediate to 1 class of
285 antimicrobials, 25.8% to 2 different classes, 8.6% to 3 different classes and 2.2% (the 2 MRSA
286 isolates) to more than 3. Table 10 reports all the raw MIC values and the MIC_{90} of the isolates for
287 each antimicrobial tested. The MIC_{90} of the all antimicrobials tested was lower than the resistance
288 breakpoint, except for penicillin, ampicillin, spyramicin and tylosin. The MIC assay (Table 10)
289 revealed that 50 (53.8%) isolates were not inhibited by the range of concentrations tested for

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290 spiramycin, which was the antimicrobial with the highest rate of resistance to. Out of 93 isolates, 21
291 (22.6%) were classified as having intermediate resistance or resistance to erythromycin, 20 (21.5%)
292 to lincomycin, 18 (19.4%) to penicillin and 17 (18.3%) to ampicillin. The nitrocefin-based method
293 detected a total of 18 (19.4%) isolates producing β-lactamase: 15 of them were also phenotypically
294 resistant to penicillin, while 3 showed susceptibility to this drug.

295 In estimating the occurrence of genes responsible for antibiotic resistance by PCR analysis, *blaZ* had
296 the highest frequency (46.2%). Additionally, we investigated the presence of *erm* genes and *mec*
297 genes that can confer resistance to erythromycin and methicillin, respectively. The genotypic results
298 (Figure 1) showed that the resistance rate to erythromycin was 32.3% and the most frequently
299 detected erythromycin resistance gene was *ermC* (21.5%), followed by *ermB* (10.8%); the prevalence
300 of methicillin-resistant *S. aureus* strains was low among the isolates analysed as only 2 (2.2%) strains
301 harboured *mecA*.

302 Figure 2 shows the association between the occurrence of genes conferring antibiotic resistance (x-
303 axis) and lab-tested phenotypic resistance to antimicrobials (y-axis). The association was calculated
304 as the sum of co-occurrences of genetic and phenotypic resistance to antibiotics normalized over
305 sample size (Buzydlowski, 2015). The molecular detection of the antibiotic resistance genes was not
306 always directly proportional to the phenotypic expression of these genes (Figure 2). The gene
307 responsible for resistance to penicillin (*blaZ*) was the most prevalent (46.2%), but only 19.4% of the
308 strains analysed demonstrated phenotypic resistance to this drug; the same percentage (19.4%) of
309 isolates was reported to be resistant due to a positive nitrocefin test result. All isolates that
310 demonstrated phenotypic resistance to penicillin or β-lactamase activity carried the *blaZ* gene. In
311 addition, 32.3% of the isolates were positive for *erm* genes but the phenotypic results showed that 21
312 (22.6%) of the 93 strains analysed were resistant (5.4%) or intermediate (17.2%) to erythromycin; 10
313 (10.8%) of the intermediate-resistant strains to erythromycin were negative for *ermB* or *ermC*. The
314 two phenotypically oxacillin-resistant strains were the only ones that harboured *mecA*.

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315 Figure 3 shows the comparison of penicillin MIC distribution and frequency of *blaZ* positive and
316 negative isolates. The distribution of *blaZ* positive MIC is scattered along the dilution range, without
317 bimodal distribution and with most of the isolates (23) having the lower MIC. The *blaZ* negative
318 isolates are gathered in the last 2 MIC dilution with a clear unimodal distribution. Figure 3 displays
319 also the comparison of erythromycin MIC distribution and frequency of *erm* positive and negative
320 isolates. In this case, *erm* positive isolates show a bimodal distribution having as cut-off the resistance
321 breakpoint, while *erm* negative isolates are distributed only behind the resistance cut-off with the
322 major frequency at 0.5 μg/mL.

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323 **DISCUSSION**

324 Of the 93 *S. aureus* strains analysed, only 2 European isolates harboured *mecA*; none were positive
325 for *mecC,* a recently identified *mecA* homologue detected in humans and in a wide range of domestic
326 and wild animals from different European countries (Schlotter et al., 2014). Our results confirmed
327 the low prevalence of MRSA among *S. aureus* strains collected from bovine mastitis samples (da
328 Costa Krewer et al., 2015; Hendriksen et al., 2008; Luini et al., 2015; Silva et al., 2013). The β-lactam
329 antibiotics have been largely used to treat *S. aureus* mastitis for several decades, but their efficiency
330 is reduced by bacterial β-lactamases. The *blaZ* gene, which encodes the β-lactamase and confers
331 resistance to penicillin (Olsen et al., 2006), was the most frequently detected resistance gene, found
332 in 43 strains. Out of these, 21, including the 2 MRSA isolates, were reported to be phenotypically
333 resistant to penicillin based on the MIC or nitrocefin-based test results. Therefore, the remaining 50%
334 of the *blaZ* positive isolates was phenotypically susceptible to penicillin, in agreement with previous
335 results reported by Ruegg and collaborators (2015). Haveri et al. (2005) suggested that the occurrence
336 of isolates with phenotypic resistance to a certain antibiotic could be not always proportional to the
337 presence of the corresponding resistance gene. Considering the isolates susceptible to penicillin but
338 positive for *blaZ* as potentially resistant (Haveri et al., 2005), our results confirmed that resistance to
339 penicillin was the most frequently observed resistance mechanism, though with a lower prevalence

340 than the over 60% reported by Malinowski and collaborators (2002, 2008). On the other hand, looking
341 at the comparison of penicillin MIC distribution and frequency of *blaZ* positive and negative isolates,
342 most of the isolates had the lowest MIC. This discrepancy between phenotypic and genotypic results
343 may demonstrate that the detection of genes does not necessarily implicate their expression; indeed,
344 the percentage of isolates phenotypically resistant to penicillin was in agreement with previous results
345 (Ruegg et al., 2015), reporting that the resistance rate to this drug has declined (Makovec and Ruegg,
346 2003), even with differences among geographical areas. Previous studies demonstrated that the
347 occurrence of phenotypically penicillin-resistant *S. aureus* strains was higher in Argentina (40%;
348 Gentilini et al., 2000) than in Germany (17%; Tenhagen et al., 2006) and in the United States (10%;
349 Anderson et al., 2006); accordingly, we found higher resistance rates in Argentina and Germany when
350 compared to New York State, but the rates were overall lower (12.5% in Argentina, 5.9% in Germany
351 and 0% in New York State).
352 Of 18 phenotypically penicillin-resistant isolates, 17 were not inhibited even by the highest
353 concentration of ampicillin tested, in accordance with previous studies (El Behiry et al., 2012;
354 Jagielski et al., 2014). This outcome could be explained by the presence of *blaZ* in all these strains,
355 since penicillin as well as ampicillin are inactivated by the β-lactamase encoded by *blaZ*. Among
356 them, 3 were negative for the β-lactamase test: this discrepancy could be due to the lower sensitivity
357 of the nitrocefin test compared to MIC assay and PCR analysis for *blaZ* gene (Ferreira et al., 2017).
358 All the 93 isolates analysed in this study were susceptible to the association of amoxicillin and
359 clavulanate with a very low MIC_{90} (0.5 µg/mL). Considering the uncertainties connected to the
360 laboratory methods for the detection of β-lactamase-producing *S. aureus* strains, the use of
361 amoxicillin and clavulanate could be recommended when only phenotypical methods are available to
362 test resistance to penicillin, given also its demonstrated efficiency in mastitis therapy (Güler et al.,
363 2005).
364 In addition to the genes responsible for resistance to β-lactams, we investigated the presence of the
365 genes encoding resistance to erythromycin. We detected *erm* genes in 30 of the 93 isolates analysed

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366 and we found that *ermC* was the most prevalent gene, similarly to the results of Aarestrup and
367 Schwarz (2006), and Sawant et al. (2009). The phenotypic results showed that the MRSA strain
368 collected in Germany was positive for *ermB* and was also classified as phenotypically erythromycin-
369 resistant, while the other MRSA isolate from Italy was negative for both *erm* genes tested and was
370 susceptible to erythromycin. Of 30 isolates positive for *erm* genes, 5 were classified as having
371 complete resistance and 6 as having intermediate resistance to erythromycin; the susceptibility of the
372 remaining 19 isolates can be due to a lack of expression of methylases encoded by *erm* genes, in
373 agreement with previous studies (Fluit et al., 2001). Other 10 isolates negative for *erm* genes were
374 intermediate resistant to erythromycin, but they were very close to the CLSI breakpoints; therefore,
375 we cannot discard the possibility that some of them could be considered as susceptible.
376 Surprisingly, among the macrolides tested, we found a high number of isolates resistant to spiramycin
377 but susceptible to erythromycin, while usually the genes responsible for resistance to erythromycin
378 usually confer resistance also to other macrolides. The possibility of an uncorrected resistance
379 breakpoint should be taken into account, and the MIC distribution could be helpful to analyse the
380 data. Spiramycin MIC results (Table 10) show a bimodal distribution with an epidemiological cut-off
381 at 4 µg/100 mL, which seems to split the isolates in 2 different phenotypical populations, and
382 corresponds to the resistance breakpoint. A greater number of isolates and dilution points in the area
383 of resistance would be to assess the accuracy of the breakpoint. Therefore, a possible bias due to
384 incorrect resistance breakpoint could not be excluded even if the epidemiological cut-off is consistent
385 with the breakpoint for this set of data. Another possible explanation of this phenomenon could be
386 the presence among the isolates of other genes encoding resistance to macrolides that have not been
387 tested in this study. The emergence of macrolide resistance genes conferring resistance to spiramycin
388 but not to erythromycin has been described in *Streptococcus uberis* (Achard et al., 2008).
389 The cephalosporins, usually classified into different generations based on their antimicrobial
390 spectrum, are often used to treat mastitis in dairy ruminants (Moroni et al., 2005). Globally, there are
391 intramammary formulations of first-generation cephalosporins (cefazolin, cephalexin, cephalotin,

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392 cephalonium, and cephapirin), second-generation (cefuroxime), third-generation (cephoperazone and
393 ceftiofur) and fourth-generation (cefquinome) (Moroni et al., 2005; Oliveira and Ruegg, 2014; Ruegg
394 et al., 2015). We tested cefazolin as a first-generation cephalosporin, and cephoperazone and
395 cefquinome as third- and fourth-generation, respectively. These last 2 antimicrobials, classified by
396 World Health Organization (WHO, 2019) as highest priority critically important antibiotics
397 (HPCIAs), were tested in this study because they were included in the MIC plates used for routine
398 testing. It is important to highlight that the use of third- and fourth-generation cephalosporins should
399 be limited only to Gram negative bacteria that show resistance to antibiotics different from HPCIAs
400 (WHO, 2017). The use of other drugs, such as first generation cephalosporins or
401 amoxicillin/clavulanate, should be preferred to these cephalosporins for the treatment of
402 staphylococcal mastitis. Results showed that the 2 MRSA isolates both displayed resistance to
403 cefoperazone (MIC 8 µg/mL). Moreover, the MRSA strain isolated in Germany had a MIC of 4
404 µg/mL, classified as intermediate, to cefazolin and cefquinome, while that isolated in Italy had a MIC
405 of 2 µg/mL, classified as susceptible, to both drugs. The 2 MRSA isolates were resistant to oxacillin
406 with a MIC value >4 µg/mL, outside of the dilution range. These data highlight that only oxacillin or
407 cefoxitin should be used to assess phenotypically the presence of MRSA, confirming the detection of
408 *mecA* gene, as advised by CLSI (CLSI, 2013). However, bovine mastitis caused by *S. aureus* strains
409 positive for *mecA* gene and treated by administration of cephalosporins show clinical outcomes with
410 low probability of cure (Pol and Ruegg, 2007; Oliveira and Ruegg. 2014; Krömker et al., 2017). For
411 this reason, antibiotic treatment decisions should be based not only on the diagnosis of the mastitis
412 causative agents obtained by microbiological and sensitivity test results but also on the identification
413 of animals with high healing prospects (Krömker et al., 2017). The MIC_{90} was lower than the
414 resistance breakpoint for the majority of the antimicrobials tested. This outcome was expected, in
415 agreement with previous study (Gentilini et al., 2000; Ruegg et al., 2015). Therefore, for many
416 antimicrobials, we selected a dilution range wider in the lower side and narrow in the upper part, to
417 possibly detect the presence of bimodal distribution also in the susceptibility dilution range. This

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418 reduced the possibility of investigating the extent of the resistance level for some antimicrobials, as
419 lincomycin, spiramycin and tylosin.
420 The analysis of the virulence profiles of the 93 *S. aureus* strains revealed that the two adhesion factors
421 Hla and Hlb, also involved in host invasion (Moroni et al., 2011), were the most frequently detected.
422 In fact, the genes for α- and β-haemolysins were found to be widely distributed in all the six countries
423 analysed, in agreement with Aarestrup et al. (1999). All strains were positive for *hla* (100%) and 79
424 (84.9%) also carried *hlb*. The gene for α-toxin is present essentially in all *S. aureus* strains (Monecke
425 et al., 2014), including isolated from humans, while the β-toxin gene, whose activity may be important
426 in the pathogenesis of mastitis, is more frequent in bovine rather than human isolates (Larsen et al.,
427 2002).

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428 We also investigated the occurrence of the immune evasion cluster (IEC) genes, that play an
429 important role in human medicine (Baptistão et al., 2016), especially in the infections caused by
430 MRSA (McCarthy and Lindsay, 2013). This cluster comprises the staphylococcal complement
431 inhibitor gene (*scn*) and the chemotaxis inhibitory protein (*chp*), which are located on an 8-kb region
432 at the conserved 3′ end of β-hemolysin (*hlb*)-converting bacteriophages (βC-φs). The region at the
433 conserved 3′ end encodes the genes *sak, sea* or *sep* (van Wamel et al., 2006). Seven different IEC
434 types (A-G) were previously identified, based on the occurrence of *sea*, *sep*, *sak*, *chp* and *scn* genes,
435 and type B (*sak-chp-scn*) was the most prevalent (van Wamel et al., 2006). The presence of this cluster
436 in large animals was previously investigated in MRSA and methicillin-sensitive *S. aureus* (MSSA)
437 isolates from different kinds of infections in pigs and horses as well as in humans with occupational
438 exposure to pigs and horses (Cuny et al., 2015). Cuny and collaborators (2015) detected the IEC genes
439 only in MRSA from horses and horse veterinarians, probably for the genome alterations, occurring
440 in adaptation to animals and including loss or acquisition of genetic elements (Schijffelen et al.,
441 2010). In this context, we investigated the occurrence of the IEC genes in *S. aureus* isolates from
442 bovine intramammary infection. Our results showed that both MRSA did not carry these genes: the
443 one from Germany was negative for all of them, while that from Italy carried only the gene encoding

444 for enterotoxin A. On the contrary, the IEC type D was detected in one Italian and one South African
445 MSSA, carrying *scn, sak* and *sea*. This cluster type has been described as quite common in human
446 MRSA (van Wamel et al., 2006). The gene *chp* was overall absent, while *sak* was present in 31.3%
447 Argentinian and 100% South African strains. Our findings are in accordance with a recent paper
448 (Magro et al., 2017), reporting that only bovine isolates were devoid of such prophage, probably
449 because the untruncated *hlb* is necessary in ungulates for the different structure of erythrocyte
450 membranes. In contrast to our results, the IEC genes were reported to be quite frequent in a recent
451 Tunisian study on *S. aureus* strains collected from cow and ewe milk: IEC type B was predominant
452 (Khemiri et al., 2019). The *sea* gene was carried on average by half of the isolates from each country,
453 with the exception of Germany and South Africa, where the prevalence of this gene was 88.2% and
454 90.1%, respectively.

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455 **CONCLUSIONS**

456 Although it isn't straightforward to generalise to the global dairy population, given the limited study
457 size and the non-probability convenience sampling scheme, the results strengthen the knowledge on
458 the virulence and antibiotic-resistance patterns of *S. aureus* strains in dairy cows. Few specific genes
459 were frequently detected in the strains analysed, suggesting that they could be related to the ability of
460 *S. aureus* to colonize the host. The *blaZ* gene was identified in most of the isolates analysed, even
461 though the detection of this gene, as well as of *erm* genes, did not correspond with the relative
462 occurrence of phenotypic resistance; further research will be necessary to validate phenotypic
463 susceptibility testing and genotypic testing. Notwithstanding the ongoing alert on methicillin-resistant
464 *S. aureus* strains, only 2 MRSA isolates were identified in this study; all other isolates were
465 susceptible to oxacillin and the majority were also susceptible to most antimicrobials tested.
466 Therefore, the presence of highly multidrug-resistant isolates was low and the emergence of
467 widespread *S. aureus* multidrug resistance is limited to MRSA, in agreement with the above-
468 mentioned works. The results obtained from the present work show that the prevalence of

469 antimicrobial-resistant *S. aureus* strains vary depending on country and herd, but collection of
470 more comprehensive data through collaboration with a greater number of countries can provide
471 further information on the spreading of antibiotic resistance; these findings could be prospectively
472 used for further studies or meta-analysis on combined datasets, indeed. To date, the results suggest
473 that it is necessary to maintain the described antimicrobial resistance trends taking antibiotic treatment
474 decisions based on rapid diagnostic and resistance tests, and to keep an adequate level of surveillance
475 on the presence of MRSA in dairy cattle, to avoid the spreading of these strains in dairy cattle
476 population and beyond.

477 **ACKNOWLEDGMENTS**

478 The authors acknowledge Belinda Gross and Janie C. Lee (Animal Health Diagnostic Center, Cornell
479 University) for their valuable revision of the English text.

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Table 1. Source and type of *S. aureus* isolates identified in this study

Country	Total isolates per Country	Number of Farms	Type of sample	Date of isolation
Argentina	16	10	C[1]	April 2015-June 2017
Brazil	15	12	Q[2]	July 2014-May 2015
Germany	17	17	Q	May 2012-August 2016
Italy	17	15	Q	September 2012-December 2016
U.S. (New York State)	17	13	Q	January 2017-April 2017
South Africa	11	9	Q	August 2016-February 2017

[1]C: composite milk sample
[2]Q: quarter milk sample

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Table 2. Primer sequences, T_m values and sizes of PCR product for the amplification of 93 *S. aureus* isolates analysed.

Target gene	Primer sequence	Tm (°C)	Amplification size (bp)	Reference
blaZ	5'-AAGAGATTTGCCTATGCTTC-3' 3'-GCTTGACCACTTTTATCAGC-5'	50	517	Sawant et al. 2009
mecA	5'-GTAGAAATGACTGAACGTCCGATAA-3' 3'-CCAATTCCACATTGTTTCGGTCTAA-5'	56	310	McClure et al. 2006
mecC	5'-CATTAAAATCAGAGCGAGGC-3' 3'-CATTAAAATCAGAGCGAGGC-5'	52	188	Paterson et al. 2012
ermA	5'-TCTAAAAAGCATGTAAAAGAA-3' 3'-CTTCGATAGTTTATTAATATTAG-5'	52	645	Sutcliffe et al. 1996
ermB	5'-CATTTAACGACGAAACTGGC-3' 3'-GGAACATCTGTGGTATGGCG-5'	55	424	Jensen et al. 1999
ermC	5'-ATCTTTGAAATCGGCTCAGG-3' 3'-CAAACCCGTATTCCACGATT-5'	55	294	Jensen et al. 1999
chp	5'-TTTTTAACGGCAGGAATCAGTA-3' 3'-TGCATATTCATTAGTTTTTCCAGG-5'	55	404	Sung et al., 2008
sak	5'-TGAGGTAAGTGCATCAAGTTCA-3' 3'-CCTTTGTAATTAAGTTGAATCCAGG-5'	55	403	Sung et al., 2008
scn	5'-ATACTTGCGGGAACTTTAGCAA-3' 3'-TTTTAGTGCTTCGTCAATTTCG-5'	55	320	Sung et al., 2008
hla	5'-GGTTTAGCCTGGCCTTC-3' 3'- CATCACGAACTCGTTCG-5'	53	534	Salasia et al., 2004
hlb	5'-GCCAAAGCCGAATCTAAG-3' 3'-CGCATATACATCCCATGGC-5'	50	833	Salasia et al., 2004
sea	5'-TAAGGAGGTGGTGCCTATGG-3' 3'-CATCGAAACCAGCCAAAGTT-5'	56	180	Cremonesi et al., 2005

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Table 3. *S. aureus* reference strains used as positive controls in PCR reactions for detection of selected genes investigated in this study

Reference strains	Target genes
ATCC[1] 19040	*chp, hlb*
ATCC[1] 19041	*sea, hla*
ATCC[1] 19048	*blaZ, nuc, sak, scn*
ATCC[1] 700699	*mecC, ermA*
IZSLER[2] 182828/321	*ermB*
IZSLER[2] 194588/52A	*ermC*
IZSLER[2] STAU26	*mecA*

[1] Reference strains with known genotype
[2] Isolates from the collection of IZSLER (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna)

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Table 4. Phenotypic antimicrobial resistance and molecular characteristic of strains isolated in Argentina.

Argentinian Isolates[1]	Phenotypic Antimicrobial Intermediate(I) or Resistance(R)	β-Lactamase Detection	*blaZ*	*mecA*	*ermB*	*ermC*	*hlb*	*sea*	*scn*	*sak*
1	SPM[2](R)	-	-	-	-	+	+	+	-	-
2	SPM(I)	-	-	-	-	-	+	-	-	+
3	ERY[3](I), SPM(I)	-	-	-	-	+	+	-	-	-
4	ERY(R), SPM(R), LIN[4](R)	-	-	-	-	+	+	+	-	-
5	-	-	-	-	-	+	+	-	-	-
6	SPM(I)	-	-	-	-	+	+	+	-	+
7	SPM(R)	-	-	-	-	+	+	+	-	+
8	ERY(I), SPM(I)	-	-	-	-	+	-	+	-	+
9	ERY(R), SPM(R), LIN(R)	-	-	-	-	+	+	-	-	-
10	ERY(R), SPM(R), LIN(R)	-	-	-	-	+	+	+	-	+
11	ERY(I), SPM(R), LIN(R)	-	+	-	-	+	+	+	-	-
12	AMP[5](R), PEN[6](R), SPM(I)	+	+	-	-	+	+	-	-	-
13	SPM(I)	-	-	-	-	+	+	-	-	-
14	SPM(I)	-	-	-	-	+	+	+	-	-
15	ERY(R), SPM(R), LIN(R)	+	-	-	-	+	+	+	-	-
16	SPM(I)	-	+	-	-	+	+	-	-	-

[1]The isolate identification numbers correspond to those in Monistero et al. (2018).
[2]SPM: spiramycin
[3]ERY: erythromycin
[4]LIN: lincomycin
[5]AMP: ampicillin
[6]PEN: penicillin

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Table 5. Phenotypic antimicrobial resistance and molecular characteristics of strains isolated in Brazil.

Brazilian Isolates[1]	Phenotypic Antimicrobial Intermediate(I) or Resistance(R)	β-Lactamase Detection	*blaZ*	*mecA*	*ermB*	*ermC*	*hlb*	*sea*	*scn*	*sak*
17	SPM[2](R), SX-T[3](R), LIN[4](R)	-	-	-	-	-	+	+	-	-
18	SPM(R), LIN(R)	-	-	-	-	-	+	+	-	-
19	ERY[5](I), SPM(R)	-	-	-	-	-	+	-	-	-
20	AMP[6](R), PEN[7](R), SPM(I), LIN(R)	+	+	-	-	-	+	-	-	-
21	SPM(I)	-	+	-	-	-	+	-	-	-
22	AMP(R), ERY(I), PEN(R), SPM(I), LIN(R)	+	+	-	-	-	+	-	-	-
23	AMP(R), PEN(R), SPM(I), LIN(R)	+	+	-	-	-	+	-	-	-
24	SPM(I)	-	-	-	-	-	+	+	-	-
25	SPM(R), SX-T(R), LIN(R)	-	-	-	-	-	+	+	-	-
26	SPM(R)	-	+	-	-	-	+	+	-	-
27	RF[8](I), SPM(R), SX-T(R)	-	+	-	-	-	+	+	-	-
28	SPM(R), LIN(I)	-	-	-	-	-	+	-	-	-
29	-	-	+	-	-	-	+	-	-	-
30	SPM(R)	-	-	-	-	-	+	+	-	-
31	SPM(R)	-	-	-	-	-	+	+	-	-

[1]The isolate identification numbers correspond to those in Monistero et al. (2018).
[2]SPM: spiramycin
[3]SX-T: trimethoprim/sulfamethoxazole
[4]LIN: lincomycin
[5]ERY: erythromycin
[6]AMP: ampicillin
[7]PEN: penicillin
[8]RF: rifampin

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Table 6. Phenotypic antimicrobial resistance and molecular characteristics of strains isolated in Germany.

German Isolates[1]	Phenotypic Antimicrobial Intermediate(I) or Resistance(R)	β-Lactamase Detection	*blaZ*	*mecA*	*ermB*	*ermC*	*hlb*	*sea*	*scn*	*sak*
47	ERY[2](I), SPM[3](R), SX-T[4](R), LIN[5](R)	-	-	-	-	-	+	+	-	-
48	SPM(R), SX-T(R), LIN(R)	-	-	-	-	-	+	+	-	-
49	SPM(R)	-	-	-	-	-	+	+	-	-
50	ERY(I), SPM(I), LIN(R)	-	-	-	-	-	-	+	-	-
51	SPM(R)	-	-	-	-	-	+	+	-	-
52	-	-	-	-	+	-	+	-	-	-
53	AMP[6](R), CEZ[7](I), CPZ[8](R), CEQ[9](I), ERY(R), OXA[10](R), PEN[11](R), SPM(R), LIN(R)	+	+	+	+	-	+	-	-	-
54	ERY(I), SPM(R)	-	+	-	+	-	+	+	-	-
55	SPM(R)	-	+	-	+	-	+	+	-	-
56	SPM(I)	-	+	-	+	-	+	+	-	-
57	SPM(R)	-	-	-	+	-	+	+	-	-
58	SPM(I)	-	-	-	-	-	-	+	-	-
59	SPM(R)	-	+	-	-	-	+	+	-	-
60	SPM(I)	-	-	-	-	-	-	+	-	-
61	CPZ(I), SPM(I)	-	+	-	-	-	-	+	-	-
62	ERY(I), SPM(R), LIN(R)	-	+	-	-	-	-	+	-	-
63	ERY(I), SPM(R)	-	+	-	-	-	-	+	-	-

[1]The isolate identification numbers correspond to those in Monistero et al. (2018).
[2]ERY: erythromycin
[3]SPM: spiramycin
[4]SX-T: trimethoprim/sulfamethoxazole
[5]LIN: lincomycin
[6]AMP: ampicillin
[7]CEZ: cefazolin
[8]CPZ: cefoperazone
[9]CEQ: cefquinome
[10]OXA: oxacillin
[11]PEN: penicillin

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Table 7. Phenotypic antimicrobial resistance and molecular characteristics of strains isolated in Italy.

Italian Isolates[1]	Phenotypic Antimicrobial Intermediate(I) or Resistance(R)	β-Lactamase Detection	*blaZ*	*mecA*	*ermB*	*ermC*	*hlb*	*sea*	*scn*	*sak*
64	AMP[2](R), PEN[3](R), SPM[4](R)	+	+	-	-	-	+	-	-	-
65	AMP(R), GEN[5](R), PEN(R)	+	+	-	-	+	+	-	-	-
66	AMP(R), PEN(R), SPM(R)	+	+	-	-	-	+	-	-	-
67	AMP(R), PEN(R), SPM(R)	+	+	-	-	+	+	-	-	-
68	AMP(R), PEN(R), SPM(R)	+	+	-	-	+	+	-	-	-
69	GEN(R), KAN[6](R), SPM(I)	-	+	-	-	-	+	-	-	-
70	SPM(I)	-	-	-	-	-	-	-	-	-
71	SPM(I)	+	+	-	-	+	+	+	-	-
72	SPM(I)	-	-	-	-	-	+	+	-	-
73	AMP(R), PEN(R)	+	+	-	-	+	+	+	-	-
74	AMP(R), PEN(R)	-	+	-	-	-	+	+	-	-
75	-	-	+	-	-	-	+	+	-	-
76	-	-	-	-	-	-	+	+	-	-
77	AMP(R), CPZ[7](R), ENRO[8](I), OXA[9](R), PEN(R), SPM(R), LIN[10](R)	+	+	+	-	-	+	+	-	-
78	AMP(R), PEN(R)	+	+	-	-	-	+	+	+	+
79	ERY[11](I), PEN(R)	+	+	-	-	-	+	+	-	-
80	SPM(I)	-	+	-	-	-	+	+	-	-

[1]The isolate identification numbers correspond to those in Monistero et al. (2018).
[2]AMP: ampicillin
[3]PEN: penicillin
[4]SPM: spiramycin
[5]GEN: gentamicin
[6]KAN: kanamycin
[7]CPZ: cefoperazone
[8]ENRO: enrofloxacin
[9]OXA: oxacillin
[10]LIN: lincomycin
[11]ERY: erythromycin

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Table 8. Phenotypic antimicrobial resistance and molecular characteristics of strains isolated in U.S. (New York State).

American Isolates[1]	Phenotypic Antimicrobial Intermediate(I) or Resistance(R)	β-Lactamase Detection	*blaZ*	*mecA*	*ermB*	*ermC*	*hlb*	*sea*	*scn*	*sak*
81	SPM[2](R)	-	-	-	-	-	+	+	-	-
82	RF[3](I), SPM(I)	-	+	-	-	-	+	+	-	-
83	SPM(R)	-	-	-	-	-	+	-	-	-
84	SPM(I)	-	-	-	-	-	+	-	-	-
85	SPM(R)	-	-	-	-	-	+	-	-	-
86	SPM(R)	-	+	-	-	-	+	-	-	-
87	SPM(I)	-	-	-	-	-	+	+	-	-
88	SPM(I), LIN[4](R)	-	+	-	-	-	+	+	-	-
89	SPM(R)	-	+	-	-	-	+	-	-	-
90	ERY[5](I), SPM(R)	-	-	-	-	-	+	+	-	-
91	SPM(R)	-	-	-	-	-	-	+	-	-
92	SPM(R)	-	-	-	-	-	+	-	-	-
93	SPM(R)	-	+	-	-	-	+	+	-	-
94	SPM(R)	-	+	-	-	-	+	+	-	-
95	SPM(R)	-	-	-	-	-	-	+	-	-
96	SPM(R)	-	+	-	-	-	+	-	-	-
97	-	-	-	-	-	-	+	-	-	-

[1]The isolate identification numbers correspond to those in Monistero et al. (2018).
[2]SPM: spiramycin
[3]RF: rifampin
[4]LIN: lincomycin
[5]ERY: erythromycin

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Table 9. Phenotypic antimicrobial resistance and molecular characteristics of strains isolated in South Africa.

South African Isolates[1]	Phenotypic Antimicrobial Intermediate(I) or Resistance(R)	β-Lactamase Detection	*blaZ*	*mecA*	*ermB*	*ermC*	*hlb*	*sea*	*scn*	*sak*
98	SPM[2](I)	-	-	-	-	-	+	+	-	+
99	ERY[3](I), SPM(R)	-	-	-	+	-	-	+	-	+
100	SPM(R), LIN[4](I)	-	-	-	+	-	-	+	-	+
101	AMP[5](R), ERY(I), PEN[6](R), SPM(R)	-	+	-	-	-	+	+	-	+
102	SPM(R)	-	-	-	+	-	+	+	-	+
103	SPM(R)	+	+	-	-	-	+	+	+	+
104	AMP(R), PEN(R), SPM(I)	-	+	-	-	-	-	+	-	+
105	SPM(R)	-	-	-	-	-	-	+	-	+
106	SPM(I)	-	-	-	-	-	+	+	-	+
107	ERY(I), SPM(I)	-	-	-	+	-	+	+	-	+
108	AMP(R), ERY(I), PEN(R), SPM(R)	+	+	-	-	-	+	-	-	+

[1]The isolate identification numbers correspond to those in Monistero et al. (2018).
[2]SPM: spiramycin
[3]ERY: erythromycin
[4]LIN: lincomycin
[5]AMP: ampicillin
[6]PEN: penicillin

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Table 10. Minimum inhibitory concentrations and MIC_{90} of the 16 antimicrobial agents tested for the 93 *Staphylococcus aureus* isolates.

	Number of Staph. aureus isolates with MIC (µg/mL)											
Antimicrobials[1]	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	MIC_{90}	Breakpoints reference
Amoxicillin-clavulanic acid[2]			82	7	2	1	1				0.5	CLSI vet01-S2 (2013)
Ampicillin		66	10	3	7	1	3	2		1	1	CLSI vet01-S2 (2013)
Cefazolin		16	22	48	5	1	1				0.5	CLSI vet01-S2 (2013)
Cefoperazone			2	16	23	49	1	2			2	Feβler et al., 2012
Cefquinome		2	22	35	30	3	1				1	Lang et al., 2002
Enrofloxacin		88	3	1	1						0.125	CLSI vet01-s2 (2013)
Erythromycin		3	9	60	13	2	1		5		1	CLSI vet01-s2 (2013)
Gentamicin					91	1				1	1	CLSI vet01-s2 (2013)
Kanamycin						76	14	2		1	4	CASFM vet 2018
Lincomycin					60	13	1	1	18		>8	CASFM vet 2018
Oxacillin		38	37	15	1			2			0.5	CLSI vet01-s2 (2013)
Penicillin	65	10	3	2	1	3	2	4			1	CLSI vet01-s2 (2013)
Rifampin					91	2					1	CLSI vet01-S2 (2013)
Spiramycin					11	32		50			>4	CASFM vet 2018
Trimethoprim-sulfamethoxazole[3]			87		1			5			0.25	CLSI vet01-s2 (2013)
Tylosin			2	4	28	59					2	Simjee et al, 2011

[1]The dilution ranges tested for each antibiotic are those within the shaded area. Values situated above the highest concentration tested indicate the number of isolates with a MIC greater than the highest dilution, values situated at the lower dilution tested indicate the number of isolates with an MIC lower o equal to last dilution of antimicrobial. Resistance breakpoints are indicated with a vertical line to the right of the breakpoint value; intermediate breakpoints are indicated with a vertical line to the left side of the breakpoint value.
[2]In the shaded area is reported the concentration of amoxicillin. Amoxicillin/clavulanic acid concentration ratio is 2:1.
[3]In the shaded area is reported the concentration of trimethoprim. Trimethoprim-sulfamethoxazole concentration ration is 1:19

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Figure 1. The occurrence of the antimicrobial resistance genes in 93 *S. aureus* isolates from different countries.

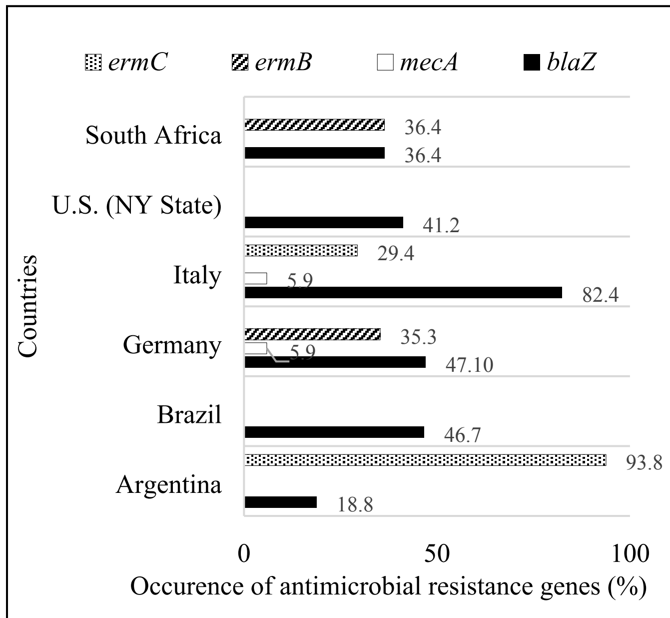

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Figure 2. Comparison of phenotypic and genotypic antimicrobial resistance for selected genes in 93 *S. aureus* isolates from different countries.

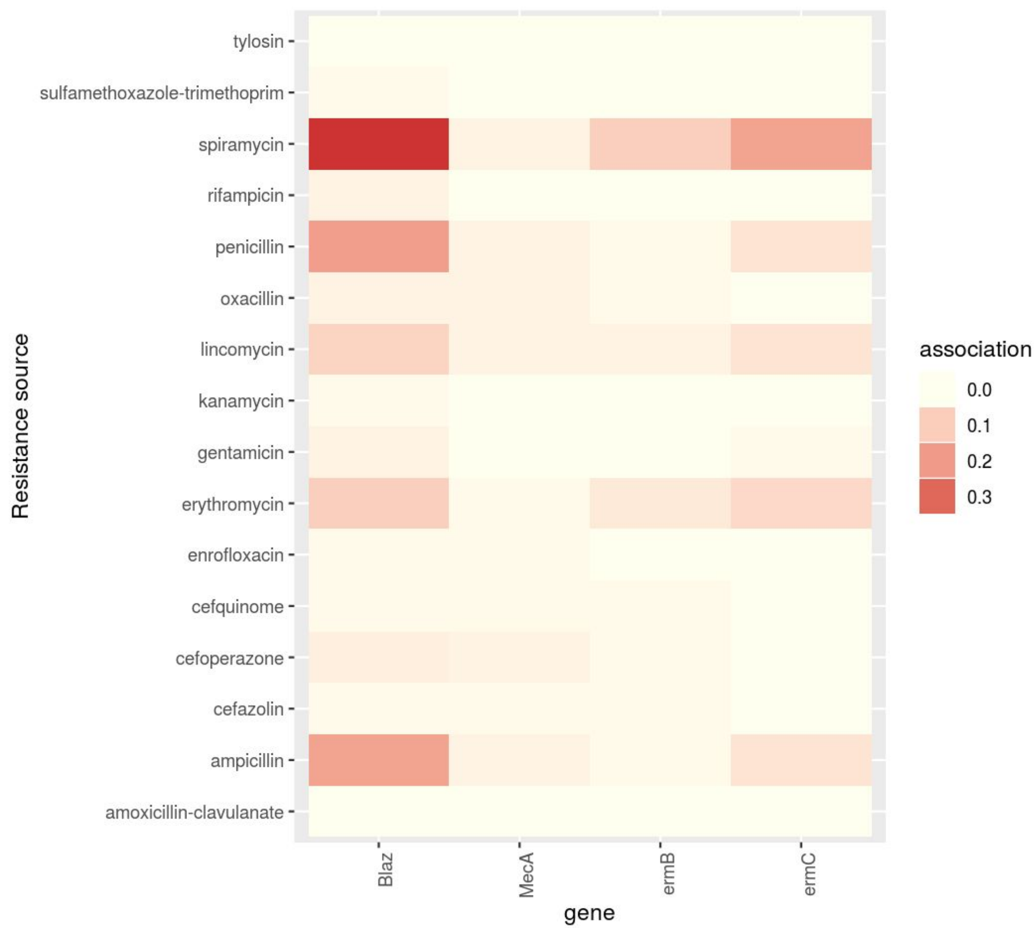

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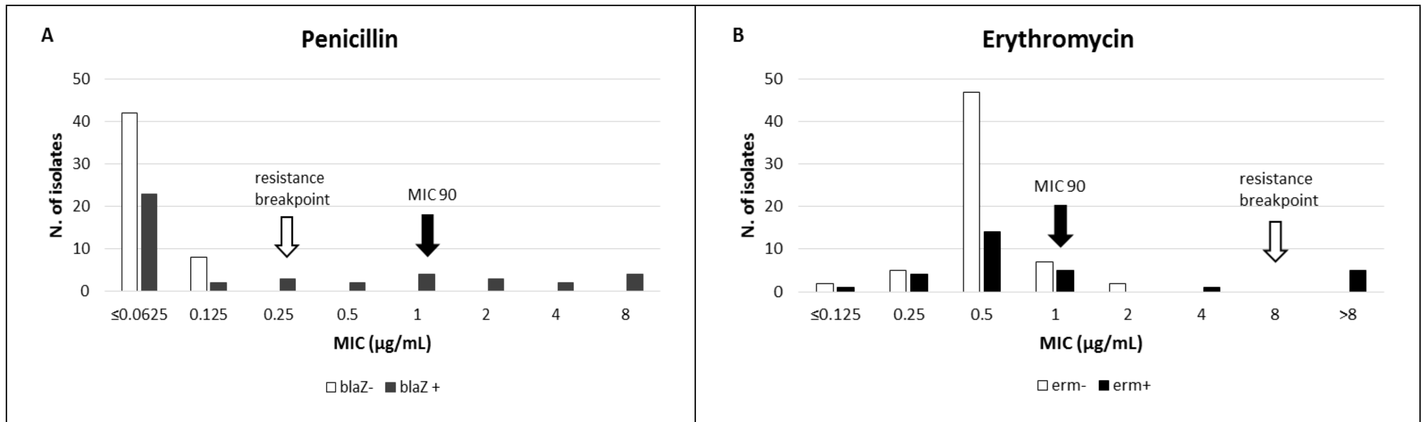
Figure 3. Comparison of penicillin MIC distribution and frequency of *blaZ* positive and negative isolates (figure A), and of erythromycin MIC distribution and frequency of *erm* positive and negative isolates (figure B). On the x-axis are displayed MIC values and on y-axis the number of isolates. The white arrows show the resistance breakpoint, the black arrows the MIC_{90} value.



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Reviewer: 2

Comments to the Author
V. Monistero and A. Barberio et al. provide a revision to their manuscript detailing their studies of antimicrobial resistance profiling and genotyping of 93 bovine <i>Staphylococcus aureus</i> isolates from clinical mastitis cases from dairy cattle housed on 76 farms in 6 different countries. The authors have addressed many of my concerns in this revision. I have continued concerns regarding clear statements of the target population of farms to which results can be extrapolated (only the actual study farms) and the non-random character of the source and study farms. There are continued issues with English grammar that diminish the work.

In my opinion, from an epidemiological perspective, the ability to link the strain typing results to the susceptibility data is a strength of this study. Numerous studies have shown associations between <i>S. aureus</i> genotype and carriage of antimicrobial resistance or other virulence factor genes; <i>S. aureus</i> genotype (e.g. MLST type, PFGE type, spa type or RS-PCR type) is not epidemiologically independent of antimicrobial resistance or other virulence factors (McCarthy and Lindsay BMC Microbiology 2010, 10:173; Chen et al. Adv Exp Med Biol 2016;915:81-97; Boss et al. 2016 J. Dairy Sci. 99:515–528; Leuenberger et al. 2019 J. Dairy Sci. 102:3295–3309, among many others). Like this study, most of these prior studies report results for a limited number of non-random isolates from a limited number of farms and geographic regions. We need continued surveillance to document historic and emerging antimicrobial resistance trends (Thakur and Gray 2019 Am J Trop Med Hyg. 100: 227–228). Reporting these data allows the research and health communities to continue to explore strain associated factors related to the global epidemiology of<i> S. aureus</i>. The combined results of this study and the previous study extend beyond practical mastitis management considerations, to include pathogen strain diversity and global variation in antimicrobial resistance among <i>S. aureus</i> strains, which is the main reason I think this study deserves consideration of publication despite the small sample size and issues with sample selection I will reiterate in this review.

AU: New line 456-457, the authors agree with the reviewer that the small sample size of the present study cannot be considered as representative of the bacterial strain dissemination among animal populations. However, the authors believe that these results for a limited number of isolates from a limited number of farms and geographic regions can be compared with results obtained from previous studies and further researches in order to understand if our findings can be universally applicable. Collection of more comprehensive data through collaborations among other countries can be the next steps of a greater project, that will aim to provide further information on the spreading of antibiotic resistance, as reported at line 468-472. The results of this project can help limiting the rise of resistance worldwide by the development of standards for surveillance of antimicrobial resistance.

I encourage the authors to find a way to satisfy the request to omit the genotyping results which have been previously published, but also to maintain a link between the PR-PCR results and the new antimicrobial susceptibility results presented in this manuscript. Unfortunately, the isolate identification numbers in this manuscript are not the same as those in the prior publication (Toxins 2018, 10, 247; doi:10.3390/toxins10060247). For example isolates #32-46 in the prior

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publication are from Columbia, while there are no isolates from Columbia in the current manuscript and isolates #32-46 are from Germany. A possible solution is to be consistent in isolate identification numbers across both manuscripts (i.e. in this manuscript use the original isolate identification numbers from the tables in the Toxins 2018 publication, so that readers can link the genotypes and other results associated with each isolate from the prior paper to the same isolates in this paper). In tables 4 to 9, please revise the isolate identification method to allow readers to determine the RS-PCR type of each isolate by referring to the isolate numbers in the tables in the Toxin 2018 publication.

AU: Tables 4 to 9, the isolate identification method has been revised and the strain numbers correspond to those used in the prior study (Monistero et al., 2018), as suggested.

I believe this might justify adding back sentences in the introduction describing the importance of the link between genotype and antimicrobial resistance. I am sorry to see some sentences deleted for the first draft, such as line 101 “<i>S. aureus </i>virulence and antimicrobial resistance profiles are highly associated to specific genotypes (Fournier et al., 2008)”, or part of line 106 “because of their strong association with virulence and resistance gene patterns (Käppeli et al., 2019).” I believe the potential link between strain type and susceptibility genotype and phenotype are relevant issues to address in this manuscript.

AU: new lines 119-120 and 125-126, the sentences deleted for the first draft have been added back, as suggested; more details has been provided to clarify the aim of this study linked to the previous one (Monistero et al, 2018).

This brings up an important limitation of the current manuscript and the previous publication (Toxins 2018). In my opinion, the use of the RS-PCR typing system is a limitation, because this system relies on interpretation of electrophoretic banding patterns, has not been adapted by other laboratories globally (issues with transferability of methods and interpretation of results by inexperienced labs) and may have a different discriminatory power compared to other internationally recognized typing systems for global epidemiology of <i>S. aureus</i> (e.g. MLST, spa typing, whole genome MLST; see for example the above four references). Note, higher discriminatory power is not always better; for global <i>S. aureus</i> epidemiology MLST might be sufficient. Because I understand the scope of labor and cost required to complete additional strain typing by MLST or spa typing, during the first round of reviews I did not request alternative sequence-based typing methods be added to this manuscript, although I strongly considered making this request. I believe the addition of a better-established globally comparable sequence-based typing method would strengthen this manuscript and make it of greater interest to a broader audience, but this is not required for me to recommend publication. Unlike nucleotide sequence-based typing systems (which are emerging as the global gold standard by replacing prior standards such as PFGE for epidemiological investigations, see for example US CDC typing methods) RS-PCR is applied by a small number of laboratories globally, and requires interpretation of fragment size banding patterns (Fournier et al. Res Vet Sci 2008 Dec;85(3):439-48) which may limit its transferability in global studies of pathogen epidemiology. Unfortunately, these issues were not discussed in Toxins publication that first reported the genotyping results. With removal of the genotyping methods and results from this

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manuscript, these issues are not relevant to the discussion in this "daughter" manuscript. I was compelled to share my opinion and welcome any comments in the response to this review.

AU: the authors agree with the reviewer that genotyping by RS-PCR is limited by the fact that investment in machines is necessary and the free Mahal software is required (Graber, JOVE, 2016). However, after equipment acquisition, the interpretation and resolution of RS-PCR is simple. Moreover, in the study of Cremonesi et al. (J. Dairy Sci., 2015) the authors showed that RS-PCR was an accurate, rapid, and inexpensive tool used to characterize *S. aureus* strains of bovine mammary origin and to generate results similar to those obtained from different genotyping methods: PFGE, MLST, ribotyping and spa typing. Therefore, the resolution of RS-PCR for bovine strains of S. aureus is as good as spa typing, and better than MLST and PFGE (Cremonesi et al., 2015; Boss et al., J. Dairy Sci., 2016). Other advantages of RS-PCR provided by Fournier et al. (2008) are the low costs, particularly when compared to MLST that requires seven genes to be sequenced in both directions (Enright et al., J.Clin.Microbiol., 2000).
In our opinion, the inexpensive RS-PCR genotyping on all the samples should be used for epidemiological analysis on a large number of isolates.

Interpretation is inconsistent with results – for example, Line 8 “To conclude, our study shows the evidence of the low prevalence of <i>S. aureus</i> antibiotic resistance.” This seems inconsistent with lines 287-289 “The phenotypic results showed that most (93.6%) of the isolates had intermediate resistance or resistance to at least one of the 16 antimicrobial agents tested. The percentage of resistance to 1-3 antimicrobials was 85%.” >90% of isolates resistant to at least one antimicrobial does not seems to be a low prevalence. Please clarify or revise your interpretations and conclusions.

AU: New lines 7-8, 71-72, 283-286, 466-467, the sentence in the Interpretative summary has been revised to clarify our conclusion about the level of antimicrobial resistance, as suggested. Moreover, a new sentence has been added in the Results related to *Association between Phenotypic Resistance and Resistance Genes* in order to provide more detailed information about the multiple drug resistance found in the isolates analyzed. Finally, the Conclusions have been revised according to reviewer comments and a new sentence has been added to improve the motivation of this conclusion.

Line 142 – methods – “For an antibiotic-resistance prevalence of about 10%, the 93 samples used in this study would give a standard error of the estimate of 0.031 (Table 1).” It is not clear to me why you added this sentence and why it is relevant to this study. Given the study design and sampling scheme I see no relevance to an <i>a priori </i>a variance estimate of your susceptibility data. Please delete this sentence, or if you decide to keep it in the manuscript then justify why this sentence is relevant in your response to this comment. Consider the US samples as an example. Your isolates are from 13 farms in New York State. In 2017 there were 4648 dairy farms in New York, and the majority would be classed as small to moderate sized (i.e. 88% of New York dairy farms had < 200 lactating cows) (https://www.nass.usda.gov/Publications/AgCensus/2017/Full_Report/Volume_1,_Chapter_1_State_Level/New_York/st36_1_0017_0019.pdf).

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AU: The sentence has been deleted, as suggested.

Were the 13 farms in this study randomly selected from all small to medium sized farms in New York? How was the number 13 farms determined?

AU: The farms were selected based on the QMPS (Quality Milk Production Services) client list, representing 6% of our small to moderate sized farms. Out of a total of 385 farms, 165 are classed as big sized and 220 as small to moderate sized. Of these 220 farms, 30% (66) were positive to *S. aureus*, of which 20% (13) were selected on the base of a non-probability convenience sample following the criteria descripted in the manuscript at line 131-136: to have a minimum of 120 lactating cows, to participate in monthly DHI testing or to use monthly CMT 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.

How can you make inferences to the diversity and susceptibility of <i>S. aureus </i>on dairy farms in New York State from these scant data of 17 isolates from 13 farms? Let alone suggest this represents the US. I imagine the same is true for each set of isolates from each country. I contend the isolates are a non-probability convenience sample, and this should be clearly stated in the methods section, or you should provide details on the methods for random selection of the farms in the study and how the data can be extrapolated to some target population of farms or isolates. Please do not try to suggest your isolates represent the actual diversity of S. aureus causing clinical mastitis among dairy cattle globally, the sample size is much too small and might not even represent the true <i>S. aureus</i> diversity on the 76 farms enrolled in this study.

AU: new line 136-137, actually, the isolates were selected based on a non-probability convenience sample and this stated has been added in the Materials and Methods section, as suggested.

Overall 10 out of 93 (10%) of isolates are <i>hla</i> negative – this seems a larger proportion compared to many previous reports. Your methods (line 151) suggest all isolates were characterized by hemolysis, what does this mean? Please clarify, and perhaps add a table indicating the relationship between presence of<i> hla</i> and <i>hlb</i> genes and hemolysis phenotypes observed after growth on appropriate blood agar media.

AU: new line 194, firstly, the *hla* gene was detected by PCR analysis using PCR Master Mix 2x (Thermo Scientific™, Waltham, MA USA). Ten isolates, previously negative for *hla,* have been reanalysed by PCR for the presence of *hla,* but using Phusion High-Fidelity Master Mix 2x (ThermoFisher Scientific, Walthem, MA, USA) to endure higher performance. Featuring an error rate lower than that of *Taq* previously used, these isolates have resulted to be positive for the gene encoding α-haemolysin, accordingly to their hemolysis phenotypes. The imagine of the relative amplified PCR fragments, visualized under UV transilluminator, can be found in Figure 1 below. Therefore, the results related to the prevalence of this gene have been changed in the manuscript. Particularly, all the

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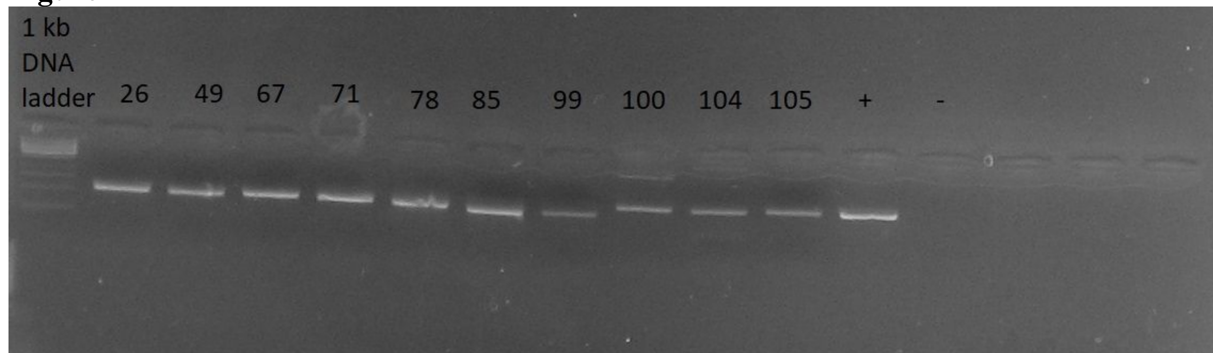
isolates were positive for *hla* as reported at new line 207-208 and, thus, the results related to the presence of this gene have been deleted in the Table 4 to 9.

Figure 1



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I find it interesting that 94% of US isolates are resistant to spiramycin (a drug which is not used in the US), while prevalence of resistance to lincosamides and other macrolides (which are used in the US) is only 6%. This warrants discussion – are the spiromycin break-points well supported for <i>S. aureus</i> from mastitis cases? How do you explain such broad phenotypic resistance to this one compound, in the absence of resistance to related compounds, especially given the discordance between spiramycin use on US dairy farms and prevalence of spiramycin resistance among US isolates?

AU: new line 376-388, we agree with the reviewer about this point because the spiramycin results were quite unusual in countries where spiramycin is not used. The manuscript was implemented with few sentences to analyze this outcome considering the overall results of all the countries, more than analyzing results of single countries. Of course, we could not provide an exhaustive explanation for this outcome, but we analyze the possible reasons for this mismatch between phenotypic and genotypic patterns of resistance to spiramycin, compared to the other macrolides tested.

Conclusion statement – line 465 and 466 – define "alarming", this seems subjective given your findings; revise or remove this statement. At what threshold of resistance prevalence should we discontinue use of an antimicrobial in a population, 25%, 50%? In human medicine > 25% resistance sounds an alarm for many pathogens. What is an alarming proportion for bovine mastitis? A 25% percent threshold is exceeded among your isolates for many antibiotics in a number of the countries. If one can use your scant data, 93 isolates from 6 countries globally, is the prevalence of spiramycin high enough to recommend it no longer be used for treatment of <i>S. aureus</i> mastitis? Revise your conclusion statements, in my opinion they are too broad and general to be supported by results from only 93 isolates.

AU: new lines 463-464 and 472-476, conclusions has been revised, as suggested. The statement not supporting an alarming increase in antimicrobial resistance has been deleted according to reviewer comments and new sentences have been added to clarify our conclusions about antibiotic resistance.

Specific comments:

Line 96 -98 – You over-interpret the data from Haveri et al., 2005; this publication is not an appropriate reference for this statement; their study did not evaluate a relationship between use of beta-lactam drugs and presence of resistance genes. I agree, the emergence of beta-lactam resistance is likely tied to beta-lactam use but the Haveri 2005 reference provides no data to support this claim. Please revise.

AU: new line 95-97, two more appropriate references have been provided for this statement, as suggested.

Grammar and sentence structure problems remain – I strongly suggest the authors engage an English language scientific editor. Examples include but are not limited to many places in the new text:

Line 75 – <i>prevalent</i> is an adjective – revise to active voice such as, <i>while the IEC gene prevalence was low in the samples analyzed</i>. Line 120-122; line 125-127; line 133-135; line 245 <i>diffused</i> should be <i>prevalent</i>; line 336-340; line 361; line 364; line 365-368; 371; conclusion paragraph.

AU: new line 74, the sentence has been revised and corrected as suggested. New lines 119-122, 123-126, 131-136, the grammar and structure of the sentences have been revised and corrected, as suggested, also with the engagement of Belinda Gross and Janie C. Lee, cited in the acknowledgments. New line 242, "diffused" has been replaced with "prevalent", as suggested. New lines 330-335, 354-355, 366, the grammar and structure of these and other sentences in the manuscripts have been revised and corrected as suggested, also with the engagement of Belinda Gross and Janie C. Lee.

Line 304 – delete <i>still</i>

AU: new line 300, "still" has been deleted, as suggested.

Line 307 – 309 – thank you for this clarification and the co-occurrence reference. Perhaps add the reference (Buzydlowski, 2015) to the end of this sentence at line 309.

AU: new line 305, the reference has been added, as suggested.

Line 399 – "be effective against <i>S. aureus </i>β-lactamase producing strains, as demonstrated in this study." Your study does not demonstrate efficacy (effectiveness) of these compounds for treatment of mastitis as some might interpret from how this sentence is written – revise this sentence to clarify.

AU: new line 400-402, the sentence has been revised and corrected, as suggested.

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Line 403 – delete “<i>of course</i>” – numerous publications report some <i>mecA</i> positive isolates can be phenotypically susceptible to oxacillin, so “of course” is not correct here.

AU: new line 405, “of course” has been deleted as suggested.

Multiple places in list of references where <i>S. aureus</i> is not italicized.

AU: *S. aureus* has been italicized in the list of references, as suggested.

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