Characterization of *Staphylococcus epidermidis* clinical isolates from hospitalized patients with bloodstream infection obtained in two time periods

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Background. In recent years *Staphylococcus epidermidis* has been considered an important and frequent causative agent of health care-associated infections (HAIs), increasing the costs of hospitalization, morbidity, and mortality. Antibiotic resistance and biofilm formation are the most important obstacles in the treatment of infections caused by this microorganism. The aim of this work was to determine the most prevalent STs, as well as the antibiotic resistance profile and biofilm formation of *S. epidermidis* clinical isolates obtained from hospitalized patients in two hospitals in Acapulco, Guerrero in two time periods. **Methods.** Twenty methicillin-resistant *S. epidermidis* strains isolated from patients with bacteremia in two hospitals in two time periods were analyzed. Identification and antibiotic susceptibility were performed using the Vitek automated system. Molecular confirmation of the identification and methicillin resistance was performed by duplex PCR of the *mecA* and *nuc* genes. Biofilm production was analyzed, and the clonal origin was determined by multilocus sequence typing (MLST). **Results.** We identified 14 antibiotic resistance profiles as well as 13 sequence types (ST), including the new ST761. We also found that ST2 and ST23 were the most prevalent and, together with ST59, were found in both time periods. Seventeen of our clinical isolates were multidrug-resistant, but all of them were sensitive to linezolid and vancomycin, and this was not related to biofilm production. Additionally, we standardized a duplex PCR to identify methicillin-resistant *S. epidermidis* strains. In conclusion, *S. epidermidis* STs 2, 23, and 59 were found in both time periods. This study is the first report of *S. epidermidis* ST761. The clinical isolates obtained

in this work showed a high multidrug resistance that is apparently not related to biofilm production.

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Abstract

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23 hospitalization, morbidity, and mortality. Antibiotic resistance and biofilm formation are the

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25 this work was to determine the most prevalent STs, as well as the antibiotic resistance profile and

26 biofilm formation of *S. epidermidis* clinical isolates obtained from hospitalized patients in two

27 hospitals in Acapulco, Guerrero in two time periods.

28 **Methods.** Twenty methicillin-resistant *S. epidermidis* strains isolated from patients with

29 bacteremia in two hospitals in two time periods were analyzed. Identification and antibiotic

30 susceptibility were performed using the Vitek automated system. Molecular confirmation of the

31 identification and methicillin resistance was performed by duplex PCR of the *mecA* and *nuc*

32 genes. Biofilm production was analyzed, and the clonal origin was determined by multilocus

33 sequence typing (MLST).

34 **Results.** We identified 14 antibiotic resistance profiles as well as 13 sequence types (ST),

35 including the new ST761. We also found that ST2 and ST23 were the most prevalent and,

36 together with ST59, were found in both time periods. Seventeen of our clinical isolates were

37 multidrug-resistant, but all of them were sensitive to linezolid and vancomycin, and this was not

38 related to biofilm production. Additionally, we standardized a duplex PCR to identify

39 methicillin-resistant *S. epidermidis* strains. In conclusion, *S. epidermidis* STs 2, 23, and 59 were

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41 isolates obtained in this work showed a high multidrug resistance that is apparently not related to

42 biofilm production.

43

Introduction

45 *Staphylococcus epidermidis* is a gram-positive, facultative anaerobic bacterium that is present in

46 the skin and mucus membranes of humans and other mammals as part of the microbiota and is

47 usually considered a commensal organism (Huttenhower et al. 2012; Paharik & Horswill 2016).

48 However, nowadays it is seen as an opportunistic pathogen, since it is the most common

49 coagulase-negative staphylococci (CoNS) responsible of health care-associated infections (HAIs)

50 (Otto 2009). HAIs, formerly known as "nosocomial" or "hospital" infections, are defined as

51 infections acquired by patients while receiving medical care in health-care facilities. These

52 infections are one of the main causes of morbidity and mortality among hospitalized patients

53 (Hsu 2014). The World Health Organization (WHO) estimates that 7 out of 100 hospitalized

54 patients in developed countries, or 10 out of 100 in developing ones, will acquire at least one

55 HAI at any given time (WHO 2020). The most important HAIs, according to frequency and

56 severity, are those related to medical procedures and devices, like surgical site infections and

57 catheter-associated infections. Around 50-70% of all cases of HAIs are associated with medical

58 devices (Bryers 2008; Guggenbichler et al. 2011).

59 *S. epidermidis* has been reported to be the main cause of central line-associated bloodstream

60 infections, the second-most-common cause of surgical site infections, and the third-most-

61 common cause of all HAI, becoming an important human pathogen that affects preferentially

62 immunocompromised, long-term hospitalized and seriously sick patients (Schoenfelder et al.

63   2010; Sievert et al. 2013). Even though infections caused by *S. epidermidis* are usually non-life-

64   threatening, probably because of the lack of classic virulence factors, the danger resides in the

65   fact that they are extremely difficult to treat due to biofilm formation and antibiotic resistance

66   (Otto 2009).

67   Biofilm production is the main virulence factor of *S. epidermidis*; particularly, its ability to form

68   it on indwelling medical devices is critically important in nosocomial bacteremia and infection of

69   prosthetic medical devices. The biofilm is a mixture of several adhesive molecules, including

70   polysaccharide intercellular adhesin (PIA), proteinaceous factors (Bhp, Aap, and Embp), teichoic

71   acids, and extracellular DNA; however, it has been shown that not all isolates encode factors

72   involved in biofilm formation. For example, the operon responsible for synthesizing PIA

73   (*icaADBC*) is present in most clinical isolates, but some clinically relevant isolates are PIA

74   negative (Fey & Olson 2010; Foster 2020). Biofilm keeps bacteria bound to inert surfaces,

75   allows them to escape the host's immune system, and protects them against antibiotics (Gotz

76   2002). Besides the protection against antibiotics conferred by the biofilm, *S. epidermidis* has

77   acquired genes that confer resistance to several antibiotics, including rifamycin, gentamicin,

78   fluoroquinolones, and erythromycin, among others. The most important antibiotic resistance is

79   methicillin resistance conferred by the *mecA* gene, which encodes a penicillin-binding protein

80   (PBP2a) that has a low affinity for beta-lactam antibiotics (Hiramatsu et al. 2001) and is

81   contained within the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) ((IWG-SCC) 2009;

82   Chambers 1997). It has been reported that approximately 80% of the isolates from device-

83   associated infections are resistant to methicillin, which is the antibiotic of first choice against

84   these infections (Diekema et al. 2001; Kozitskaya et al. 2004). In addition, healthcare associated

85   strains have high antibiotic resistance, being resistant to most of the antibiotics commonly used

86   (Chabi & Momtaz 2019).

87   The genome of *S. epidermidis* is prone to horizontal gene transfer of mobile genetic elements and

88   to chromosomal recombination, which gives rise to the emergence of different clones (Miragaia

89   et al. 2008; Miragaia et al. 2007). PFGE has been considered the gold standard for *S. epidermidis*

90   molecular typing since it is a clinically valuable tool for evaluating short time epidemiological

91   infections and is still the most discriminating method. However, several studies that used PFGE

92   for the classification of nosocomial *S. epidermidis* isolates have shown that there is an extensive

93   diversity among populations. Due to this, the usefulness of this method to evaluate *S.*

94 *epidermidis* in long-term global epidemiology has been debated (Widerstrom et al. 2009). To aid

95 in this subject, the genetic relatedness between different isolates is analyzed by MLST, a

96 technique based on sequencing of conserved housekeeping genes. This technique allows the

97 comparison of isolates, even from different countries as well as the naming of clones from all

98 over the world. Nowadays, a widely recognized MLST scheme and database have been

99 developed for *S. epidermidis* (Thomas et al. 2007).

100 It has been shown that *S. epidermidis* clones that persist and spread within the hospital setting,

101 can arise in the community or be endemic in the hospital (Brito et al. 2009; Widerstrom et al.

102 2006). Previously, we showed that *S. epidermidis* clinical isolates obtained in the General

103 Hospital of Acapulco from patients diagnosed with nosocomial infections, formed two clusters

104 that were able to persist in different nosocomial areas at least for 3 years (Castro-Alarcon et al.

105 2011). The aim of this work was to identify the ST of methicillin-resistant *S. epidermidis*

106 (MRSE) clinical isolates from two hospitals in Acapulco, Guerrero, Mexico, isolated in two

107 different periods, as well as to determine their antibiotic resistance profile and biofilm

108 production.

Materials and methods

109
110 **Study subjects**

111 Clinical isolates were obtained from patients hospitalized in different areas [neonatology (6/20),

112 pediatrics (5/20), emergency room (2/20), internal medicine (4/20), intensive care unit (2/20),

113 and gynecology (1/20)] of two tertiary care hospitals in Acapulco, Gro., Mexico in two periods:

114 Acapulco General Hospital, between 2003 and 2004, and Vicente Guerrero Hospital in 2017. All

115 patients were diagnosed with bacteremia and the clinical isolates were considered the etiological

116 agents of the infection by the Epidemiological Surveillance Committee of each hospital. One *S.*

117 *epidermidis* clinical isolate was obtained from each patient. All the isolates were obtained from

118 blood cultures. The study was approved by the Research Ethics Committee of the Autonomous

119 University of Guerrero (CB-001/22).

120 **Clinical isolates and susceptibility testing**

121 A total of 20 *S. epidermidis* clinical isolates were analyzed. Ten strains were isolated between

122 2003 and 2004, and ten were isolated in 2017. Isolates were grown overnight in Muller Hinton

123 agar (BD, Germany) at 35°C and stored at -80°C in 15% (vol/vol) glycerol and LB broth

124 (Dibico, Mexico). Strain identification, as well as antimicrobial susceptibility testing, were

125 performed using the Vitek®2 automated system (bioMérieux), which uses a fluorogenic

126 methodology for organism identification, as well as a turbidimetric method for susceptibility

127 testing that allows it to indicate the MIC for each antibiotic tested (Supplemental Table 1).

128 Antibiotics assayed were: benzylpenicillin, gentamicin, levofloxacin, erythromycin,

129 quinupristin/dalfopristin, rifampicin, trimethoprim/sulfamethoxazole, clindamycin, cefoxitin,

130 oxacillin, linezolid, and vancomycin. Antibiotic resistance or susceptibility classification was

131 performed according to the breakpoints established by the CLSI (M100 Performance Standards

132 for Antimicrobial Susceptibility Testing, 27th edition). Methicillin resistance was determined by

133 cefoxitin and oxacillin testing (M02-A11 document of the Clinical and Laboratory Standards

134 Institute, CLSI).

135 **Molecular confirmation**

136 Molecular confirmation of *S. epidermidis* identification and methicillin resistance was performed

137 by duplex PCR, using specific primers for the *nuc* and *mecA* genes, respectively (Table 1). The

138 *nuc* gene encodes a conserved thermonuclease present in all staphylococci species, except those

139 of the *S. sciuri* group, and shows moderate sequence diversity, which allows for species

140 identification with a 100% sensitivity and specificity using specific primers for each species

141 (Hirotaki et al. 2011).

142 To obtain total DNA, each isolate was grown overnight in BHI broth (BD, USA) at 35°C with

143 shaking. Then 1 ml of each culture was centrifuged at 13,000 rpm for 12 min, the pellets were

144 washed with 200 μl of sterile water and resuspended in 100 μl of water. The samples were frozen

145 at -20°C for 30 min, boiled for 5 min, frozen again for 5 min, and centrifuged at 13,500 rpm for

146 10 min. The supernatant was recovered, and the DNA was precipitated with 250 μl of cold

147 isopropanol. The DNA pellets were washed with 250 μl of 70% ethanol, dried, and resuspended

148 in 100 μl of water.

149 Each PCR was performed in a final volume of 50 μl, containing buffer 1X, 1.5 mM $MgCl_2$, 200

150 μM dNTPs, 0.6 μM of each oligonucleotide, 120 ng DNA, and 1 U Taq DNA polymerase

151 (Roche, USA). The conditions used were: 1 cycle at 95°C for 5 min; 30 cycles at 95°C for 1 min,

152 56°C for 1 min, and 72°C for 30 s; and 1 cycle at 72°C for 10 min. Amplicons were analyzed by

153 2% agarose gel electrophoresis. *S. epidermidis* ATCC 35984 and *S. aureus* ATCC 29213 were

154 used as positive and negative controls, respectively.

155 **Multilocus sequence typing**

156 All strains were analyzed by the MLST protocol described by Thomas *et al.* (2007) using

157 primers listed in Table 1. PCRs were performed in a 50 μl final volume, containing 1 U of

158 Phusion HF DNA polymerase (Thermo Fisher Scientific, USA), 1X HF buffer, 200 μM dNTPs,

159 0.24 μM of each oligonucleotide, and 120 ng of DNA. Conditions used were 1 cycle at 95°C for

160 3 min; 34 cycles at 95°C for 30 s, 50°C for 1 min, 72°C for 1 min; and 1 cycle at 72°C for 10

161 min. The amplified products were further purified using the PureLink PCR purification kit

162 (ThermoFisher Scientific) following the manufacturer's instructions and sequenced in an ABI

163 PRISM 310 Genetic Analyzer (Applied Biosystems) using the BigDye Terminator kit v.1.1

164 (Applied Biosystems) and primers listed in Table 1. The sequences were analyzed using the

165 SeqMan Pro 14.1 software (DNASTAR). The allelic profiles, consisting of the allele number for

166 each of the seven genes, were assigned to sequence types (STs) using the *Staphylococcus*

167 *epidermidis* MLST website (https://pubmlst.org/sepidermidis/, University of Oxford). *S.*

168 *epidermidis* ATCC 12228 was used as a control.

169 **Use of the goeBurst algorithm**

170 The clonal relationship of the isolates was analyzed using the goeBURST algorithm

171 (http://www.phyloviz.net/), which uses multilocus sequence typing data and clusters the STs

172 according to their number of single, double, and triple locus variants, and occurrence frequency,

173 in order to identify alternative patterns of descent based on the number of differences between

174 numeric profiles (Francisco et al. 2009). The profile data and the auxiliary data files containing

175 the ST and the alleles of the genes of each clinical isolate used in our work or registered in the *S.*

176 *epidermidis* database (https://pubmlst.org/sepidermidis), respectively, were uploaded as profile

177 data on the online tool. The program was run by hitting the "launch tree" button, and the tree was

178 constructed using the default settings.

179 **Biofilm quantification**

180 Biofilm quantification was performed according to Stepanovic et al., (2007). Briefly, the isolates

181 were grown overnight in Muller Hinton agar at 35°C. Then, bacteria were collected in sterile

182 distilled water adjusting to an $OD_{600}= 0.1$. Twenty μl of each culture were used to inoculate 180

183 μl of TBS broth supplemented with 1% glucose in 96-wells plates, and incubated at 35°C for 24

184 hr. The bacteria were washed 3 times with 300 μl of PBS 1X, fixed with 150 μl of methanol,

185 dried overnight, stained with crystal violet 2%, and washed 2 times. Then the biofilm was

186 resuspended with 95% ethanol and quantified at 570 nm in a Multiskan GO Microplate

187 spectrophotometer (ThermoScientific, Finland). Quantification was done three times by

188 triplicate. The strains were considered as non-producers when OD≤0.3, weak producers when

189 0.3<OD≤1.0, and strong producers when OD>1.0, as per recommendations by Stepanovic et al.,

190 (2007).

Results

Phenotypic and molecular identification of *S. epidermidis* and resistance profile

193 A total of 20 *S. epidermidis* clinical isolates identified by the Vitek system were included in this

194 study. The antibiotic resistance testing determined that all the isolates (100%) were resistant to

195 benzylpenicillin and oxacillin, 90% (18/20) were resistant to erythromycin, 80% (16/20) were

196 resistant to trimethoprim/sulfamethoxazole, and 70% (14/20) to clindamycin. None of the

197 clinical isolates was resistant to linezolid and vancomycin. Casually, the proportion of resistant

198 isolates was the same for all antibiotics in both groups of isolates, except for rifampicin, for

199 which we found 5 resistant isolates in 2003/2004 but only one in 2017. The isolates were then

200 confirmed as methicillin-resistant *S. epidermidis* by a duplex PCR detecting *mecA* and *nuc* genes

201 (Fig. 1).

202 We then determined if our clinical isolates were multidrug resistant. We found 14 resistance

203 profiles (Table 2), which include 8 to 3 of the antibiotics tested. Of all the clinical isolates, 85%

204 (17/20) were multidrug-resistant (≥3 antibiotics) (Magiorakos et al. 2012), of these, 20% (4/20)

205 were resistant to eight antibiotics, and 25% (5/20) were resistant to seven antibiotics.

Identification of *S. epidermidis* clinical isolates ST

207 Our molecular analysis identified 13 STs, indicating a high genotypic diversity. Ten of these STs

208 correspond to single isolates, while two correspond to four isolates and one to two isolates (Table

209 3). The most represented STs were ST2 and ST23, which include 4 strains each, and together

210 with ST640 have been found in Mexico. One of the STs identified was new, not reported

211 previously. The information of this new ST was sent to the curator of the MLST database and

212 was assigned the number 761. ST761 (arcC 28, aroE 3, gtr 5, mutS 5, pyrR 11, tpiA 4, yqiL 4)

213 (Table 3) isolated in 2017 is resistant to benzylpenicillin, oxacillin, erythromycin, clindamycin,

214 trimethoprim/ sulfamethoxazole, gentamicin, and non-biofilm producer (Table 2).

215 STs 23, 2, and 59 were isolated in both periods of time, while the rest were isolated in either

216 period (Fig. 2).

Clonal relationships

218   In order to establish the clonal relationships of our isolates, we used the algorithm goeBURST.

219   As seen in Fig. 3, STs 2 and 23, previously find in Mexico, are related to ST135, differing in two

220   and one locus variant, respectively. ST640, the other ST previously found in Mexico, is distantly

221   related to them. Interestingly, the new ST761 is more related to ST640, although they differ in 4

222   locus variants. STs 5 and 59 are related to ST57, differing only in one locus variant, as well as

223   STs 89 and 259.

224   **Biofilm production**

225   As mentioned before, biofilm production is important for the establishment of the infection. We

226   quantified the biofilm production of our isolates. Our results show that eight isolates were weak

227   producers, and 11 were non-producers (Table 2), curiously only one isolate was a strong

228   producer. Of the weak producers, one isolate belongs to ST2 and two belong to ST23, the most

229   frequent STs (Table 2). Both strains of ST59 were also weak producers, while the strain of the

230   new ST identified was a non-producer. The statistical analysis of the OD of the biofilm

231   production showed statistically significant differences among the three categories ($p < 0.001$) (Fig.

232   4).

233   **Discussion**

234   Nowadays, *S. epidermidis* is a common HAI etiological agent that causes mainly catheter-related

235   bloodstream infections. In the present study, we used MRSE clinical isolates identified by the

236   microbial identification system Vitek2 (bioMérieux), however, the identification rate of this

237   system is 93.7% for clinical isolates (Jin et al. 2011). In order to corroborate the identification,

238   we innovated a duplex PCR to amplify the *nuc* and *mecA* genes, using oligonucleotides

239   previously published (Table 1). This PCR proved to be a fast and easy way to identify MRSE

240   clinical isolates and has been introduced as the method of choice for *S. epidermidis* identification

241   at the participant hospitals.

242   The clinical isolates were obtained from blood cultures of hospitalized patients diagnosed with

243   bacteremia in the city of Acapulco, Guerrero. These isolates were obtained 13 years apart in two

244   different hospitals. We identified 13 STs, five corresponding to isolates obtained in 2003/2004 at

245   the Acapulco General Hospital, represented by one strain each, and five corresponding to strains

246   isolated in 2017 at the Vicente Guerrero Hospital also represented by one strain each. Of the

247   strains obtained in 2017, ST761 has not been previously reported, making this the first report of

248   this new ST. Interestingly, ST761 was isolated in 2017, which could be due to it being an

249 emerging ST or just not previously identified. The other three STs correspond to strains isolated

250 in both time periods and are the most represented: ST2 and ST23, with four strains each, and

251 ST59, with two strains. These STs were found in both hospitals but their distribution over time

252 was different, three strains of ST23 were isolated in 2004 and one in 2017, while the opposite

253 was found for ST2. On the other hand, one strain of ST59 was isolated in 2004 and one in 2017.

254 Our results show that STs 2, 23, and 59 are the most prevalent and persistent in hospital settings

255 in Acapulco, Gro., Mexico. This is consistent with previous reports that have found ST2 to be the

256 most predominant ST in hospital settings in China (Du et al. 2013; Li et al. 2009), Brazil (Iorio et

257 al. 2012), Portugal (Miragaia et al. 2007), USA (Sharma et al. 2014), Germany (Kozitskaya et al.

258 2004), and Australia (Widerstrom et al. 2012). Specifically, our data are similar to those obtained

259 in Brazil by Iorio et al (2012), who found that ST2 and ST23 were the most frequent in clinical

260 isolates from hospitals in Rio de Janeiro. *S. epidermidis* STs 2 and 23 have been reported

261 previously in Mexico, along with STs 640, 46, 61, 71, 7, and 82 (Table 3) (Martinez-Melendez et

262 al. 2016; Miragaia et al. 2007). With this work we add STs 5, 57, 59, 89, 135, 173, 182, 193,

263 259, 640, and the new 761.

264 *S. epidermidis* infections are usually associated with high antimicrobial resistance. When we

265 analyzed antibiotic resistance, we found 14 different resistance profiles. Three isolates were

266 resistant to two different antibiotics, while the rest were resistant to three or more. Besides all

267 isolates being penicillin-resistant, over 70% of our isolates were resistant to erythromycin,

268 clindamycin, and trimethoprim/sulfamethoxazole; between 55% and 30% were resistant to

269 gentamicin, levofloxacin, and rifampicin; and only 5% (1/20) were resistant to

270 quinupristin/dalfopristin. This antibiotic is a streptogramin that was approved by the US-FDA in

271 1998 as a treatment option for vancomycin-resistant enterococci (Shariati et al. 2020). These

272 results show that clinical MRSE strains have a high antibiotic resistance, which could complicate

273 their treatment. On the bright side, all our clinical isolates were sensitive to linezolid, a synthetic

274 oxazolidinone recommended to treat multidrug-resistant gram-positive infections (Hashemian et

275 al. 2018). Our result is consistent with those of a systematic review and meta-analysis, which

276 reports that linezolid resistance is very low (0.3%) (Shariati et al. 2020). However, our result

277 differs from a study in Mexico in which the authors report a frequency of linezolid resistance of

278 3.6% (3/83) in *S. epidermidis* clinical isolates from patients hospitalized in Guadalajara and

279 Monterrey, and that the three isolates belonged to ST23 (Martinez-Melendez et al. 2016). In

280 accordance with this, Campanile *et al*., (2013) reported that ST23 is more capable of accepting

281 the *cfr* gene, which mediates linezolid resistance and was first identified in plasmid pSCFS1

282 (Schwarz et al. 2000). On the other hand, whole-genome sequencing (WGS) of 176 *S.*

283 *epidermidis* clinical isolates from American patients showed that *S. epidermidis* ST5 and ST22

284 are related to linezolid resistance, and the authors reported a frequency of 22% (Li et al. 2018).

285 In these isolates, the *cfr* gene is contained in plasmid pMB151a, which is present in all *S.*

286 *epidermidis* ST5 linezolid-resistant clinical isolates (Li et al. 2018).

287 Our clinical isolates were also sensitive to vancomycin, which is an antibiotic used as a first line

288 drug to treat methicillin-resistant CoNS (Shariati et al. 2020). This result is similar to that

289 reported by Martinez-Meléndez et al (2016), who found 0% (0/83) resistance to vancomycin in

290 clinical isolates of Mexican hospitalized patients. Therefore, vancomycin activity against MRSE

291 clinical isolates suggests that this antibiotic may be suitable in the combat of *S. epidermidis*

292 associated with HAIs in Mexico. Interestingly, we found no relationship between STs and

293 antibiotic resistance profile. For example, ST23 strains isolated in 2004 have resistance profiles 1

294 and 3, while the isolate from 2017 has profile 1. ST2 strains isolated in 2017 have profile 4,

295 while the isolate from 2004 has profile 1. This suggests that even though STs 23, 2, and 59 seem

296 to be persistent in Acapulco, Guerrero, there are several circulating strains.

297 Numerous studies report the ability to form biofilms as a typical feature of many nosocomial

298 isolates (Gotz 2002), however, we found that almost half of our isolates were weak producers

299 and one strong producer, while the rest were non-producers. Four of the eight weak producers

300 correspond to STs 23, 2, and 59, which were the most frequent, and the strong producer belongs

301 to ST23. The biofilm-producing clinical isolates that correspond to STs 23 and 2 showed a

302 higher antibiotic resistance, since they were resistant to eight antibiotics, while ST59 clinical

303 isolates were resistant to four and five antibiotics. Biofilm is recognized as contributing

304 substantially to increased resistance to antibiotics and innate host defense, due to structured-

305 based limited diffusion or repulsion, which limits the efficacy of the antibiotics (Otto 2006).

306 However, we did not find a relationship between biofilm formation and antibiotic resistance,

307 probably due to the small number of isolates used.

308 Despite the small number of clinical isolates, and not having identified the genes responsible for

309 biofilm production (*ica* operon, polysaccharide intercellular adhesin) in order to correlate this

310 gene with weak and strong production, important information regarding the antimicrobial

311 resistance, as well as a new ST were determined. Nevertheless, future work is necessary to

312 elucidate the role of *S. epidermidis* biofilm in antibiotic resistance.

313 In conclusion, *S. epidermidis* STs 2, 23, and 59 were present in two hospitals in different periods

314 in Acapulco, Guerrero, Mexico. The clinical isolates obtained in this work showed a high

315 multidrug resistance that is apparently not related to biofilm production. The duplex PCR

316 standardized in this work, using specific primers for the *nuc* and *mecA* genes, is a reliable

317 method to identify methicillin-resistant *S. epidermidis* that can be used to continue the

318 epidemiological surveillance of HAIs. This study is the first report of *S. epidermidis* ST761.

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507

508

Figure 1

Fig. 1. Representative gel of duplex PCR results.

Fragments corresponding to the *mecA* and *nuc* genes are shown. Lanes: MW- molecular weight marker (bp), PC- positive control (*S. epidermidis* ATCC 35984), NC- negative control (*S. aureus* ATCC 29213).
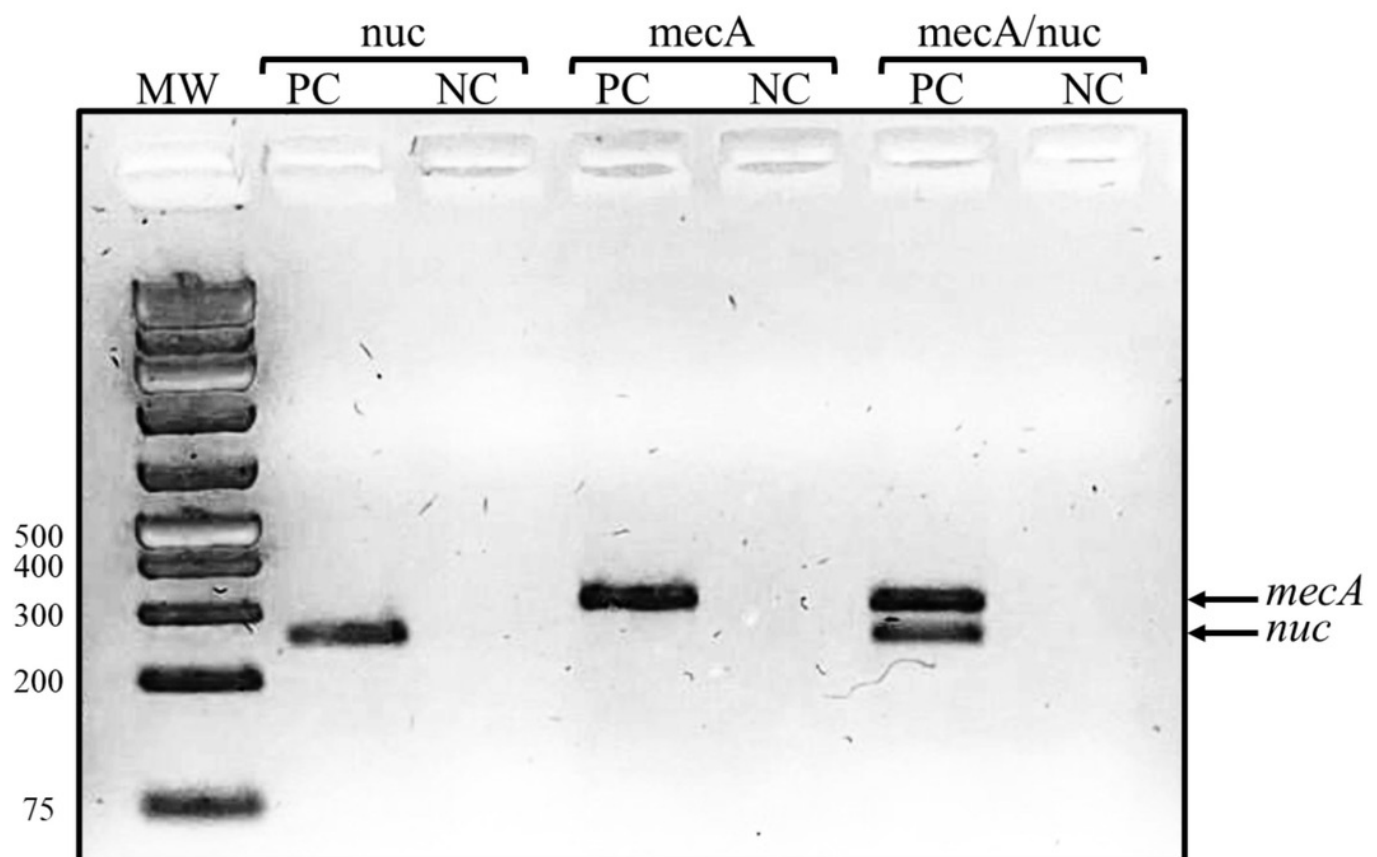
Figure 2

Fig. 2. Distribution of clinical isolates STs in time.

Venn diagram of the identified STs by year.
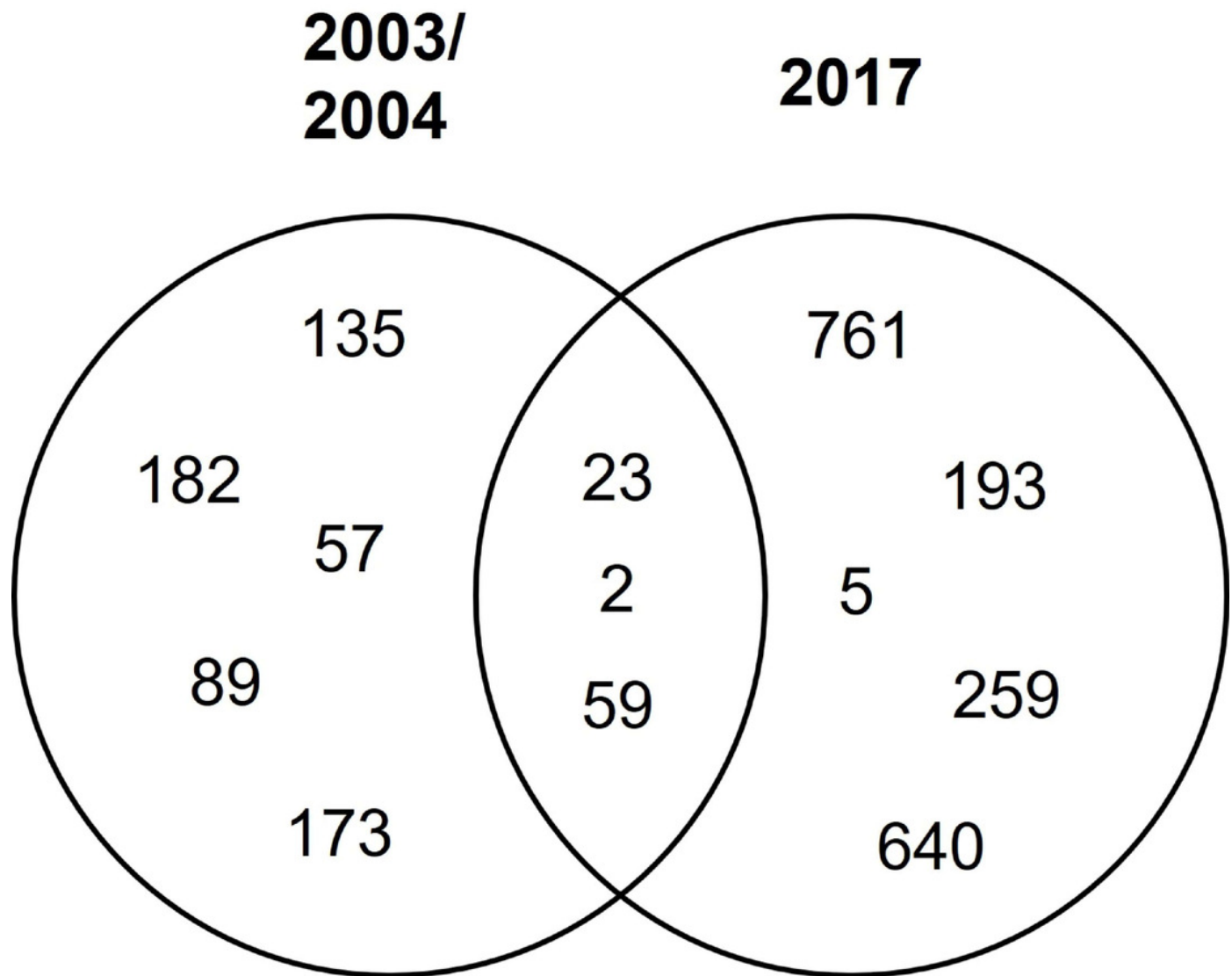
Figure 3

Fig. 3. goeBURST analysis of MLST data of *S. epidermidis* clinical isolates obtained in 2003/2004 and 2017.

Each gray circle represents a ST. Each ST is related to the others by the number of allelic differences indicated near the lines.
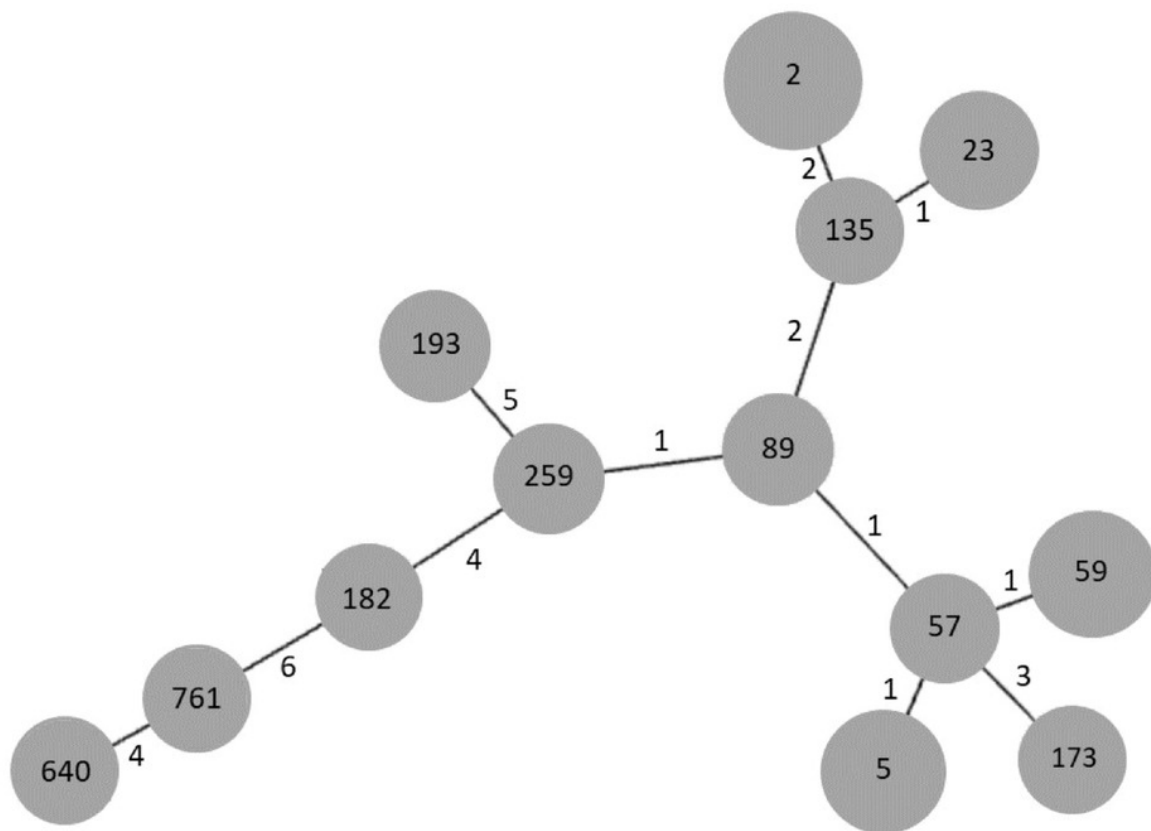
Figure 4

Fig. 4. Biofilm production.

Graph of the optical densities (OD_{570}) obtained with *S. epidermidis* clinical isolates. The bars represent the means of the ODs obtained for each category, and the error bars represent the standard deviation. Strains ATCC 35984 and ATCC 12228 were used as positive (strong producer) and negative (non-producer) controls, respectively. The p-value was calculated using t Student, and $p < 0.05$ was considered statistically significant.
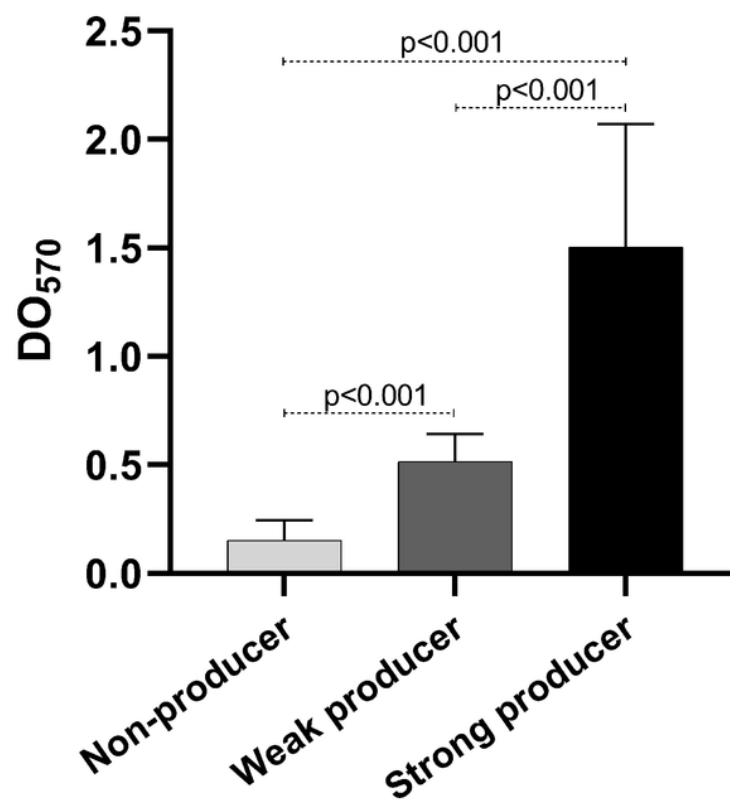
Table 1. Oligonucleotides used in this work

NA: not applicable

1   **Table 1.** Oligonucleotides used in this work

Oligonucleotide	Sequence (5´-3´)	Amplicon size (bp)	Amplicon used for allele assignment (bp)	Reference
epi-F (*nuc*)	TTGTAAACCATTCTGGACCG	251	NA	(Hirotaki et al. 2011)
epi-R (*nuc*)	ATGCGTGAGATACTTCTTCG			
mecA-F	TGGCTATCGTGTCACAATCG	310	NA	(Vannuffel et al. 1998)
mecA-R	CTGGAACTTGTTGAGCAGAG			
arcC-F	TGTGATGAGCACGCTACCGTTAG	508	465	(Thomas et al. 2007)
arcC-R	TCCAAGTAAACCCATCGGTCTG			
aroE-F	CATTGGATTACCTCTTTGTTCAGC	459	420	
aroE-R	CAAGCGAAATCTGTTGGGG			
gtr-F	CAGCCAATTCTTTTATGACTTTT	508	438	
gtr-R	GTGATTAAAGGTATTGATTTGAAT			
mutS-F3	GATATAAGAATAAGGGGTTGTGAA	608	412	
mutS-R3	GTAATCGTCTCAGTTATCATGTT			
pyr-F2	GTTACTAATACTTTTGCTGTGTTT	851	428	
pyr-R4	GTAGAATGTAAAGAGACTAAAATGAA			
tpi-F2	ATCCAATTAGACGCTTTAGTAAC	592	424	
tpi-R2	TTAATGATGCGCCACCTACA			
yqiL-F2	CACGCATAGTATTAGCTGAAG	658	416	
yqiL-R2	CTAATGCCTTCATCTTGAGAAATAA			

3   NA: not applicable.

Table 2. Characteristics and resistance profiles of MRSE clinical isolates used in this work

BP: benzylpenicillin, OX: oxacillin, EM: erythromycin, CM: clindamycin, Q/D:

quinupristin/dalfopristin, LE: levofloxacin, GM: gentamicin, TS:

trimethoprim/sulfamethoxazole, RI: rifampicin, ER: emergency room, IM: internal medicine,

ICU: intensive care unit, AGH: Acapulco General Hospital, VGH: Vicente Guerrero Hospital.

1   **Table 2.** Characteristics and resistance profiles of MRSE clinical isolates used in this work

Resistance profile	Antibiotics	Clinical isolate	ST	Biofilm production	Hospital area	Hospital	Year of isolation
1	BP, OX, EM, CM, LE, TS, RI, GM	1069	23	Strong	Neonatology	AGH	2004
		1091	2	Weak	Pediatrics	AGH	2004
		4204	23	Weak	ER	VGH	2017
2	BP, OX, EM, CM, LE, TS, RI, Q/D	1154	135	Non	Neonatology	AGH	2004
3	BP, OX, EM, CM, LE, TS, RI	1047	23	Non	Neonatology	AGH	2004
		1103	23	Non	Pediatrics	AGH	2004
4	BP, OX, EM, CM, LE, TS, GM	4205	2	Non	IM	VGH	2017
		4208	2	Non	IM	VGH	2017
		4206	2	Non	ER	VGH	2017
5	BP, OX, EM, CM, TS, GM	1126	182	Non	Neonatology	AGH	2004
		4211	761	Non	IM	VGH	2017
6	BP, OX, EM, CM, LE, TS	4212	5	Weak	ICU	VGH	2017
7	BP, OX, EM, TS, GM	1032	89	Non	Neonatology	AGH	2003
8	BP, OX, EM, CM, TS	4202	59	Weak	Pediatrics	VGH	2017
9	BP, OX, EM, CM	1042	59	Weak	Neonatology	AGH	2004
10	BP, OX, EM, TS	1161	173	Weak	Gynecology	AGH	2004
11	BP, OX, EM, GM	4214	259	Weak	Pediatrics	VGH	2017
12	BP, OX, GM	585	57	Weak	Pediatrics	AGH	2003
13	BP, OX, EM	4201	640	Non	IM	VGH	2017
14	BP, OX, TS	4210	193	Non	ICU	VGH	2017

5   BP: benzylpenicillin, OX: oxacillin, EM: erythromycin, CM: clindamycin, Q/D: quinupristin / dalfopristin, LE:

6   levofloxacin, GM: gentamicin, TS: trimethoprim / sulfamethoxazole, RI: rifampicin, ER: emergency room, IM:

7   internal medicine, ICU: intensive care unit, AGH: Acapulco General Hospital, VGH: Vicente Guerrero Hospital.

Table 3. MRSE sequence types identified in this study and countries where they have been found

UN: Unknown, NA: Not applicable. a Countries and number of isolates according to the MLST database: https://pubmlst.org/sepidermidis/ (until February 2022). b New ST found in this work.

1 **Table 3.** MRSE sequence types identified in this study and countries where they have been

2 found

ST	MLST profile (*arc, aroE, gtr, mutS, pyrR, tpi, yqiL*)	Clinical isolates	Countries (number of isolates)[a]
2	7,1,2,2,4,1,1	1091, 4205, 4206, 4208	Argentina (2), Brazil (18), Bulgaria (1), Cape Verde (2), Colombia (1), Denmark (2), France (1), Germany (9), Greece (2), Hungary (2), Iceland (2), Iran (5), Ireland (1), Italy (2), Japan (3), Mexico (2), Netherlands (1), Poland (2), Russia (1), South Africa (4), South Korea (19), Spain (2), UN (2), Uruguay (2), USA (12)
5	1,1,1,2,2,1,1	4212	Bulgaria (1), Cape Verde (1), Denmark (2), Germany (10), Iceland (1), Iran (3), Ireland (1), Norway (1), Poland (2), Russia (2), South Korea (2), UN (2), USA (5)
23	7,1,2,1,3,3,1	1047, 1069, 1103, 4204	Argentina (1), Brazil (5), Germany (2), Greece (1), Hungary (1), Iceland (1), Mexico (1), Poland (1), Portugal (1), Uruguay (1), Russia (4), USA (1)
57	1,1,1,1,2,1,1	585	Germany (1), India (2), Portugal (1)
59	2,1,1,1,2,1,1	1042, 4202	Brazil (8), Germany (5), Greece (1), India (1), Ireland (1), Russia (21), South Africa (1), South Korea (4)
89	1,1,2,1,2,1,1	1032	Brazil (1), China (2), Denmark (1), Iceland (1), Netherlands (1), Norway (1), Portugal (2), USA (2)
135	7,1,2,1,3,1,1	1154	UN (1)
173	1,6,6,1,2,1,10	1161	Germany (1), Poland (1), South Korea (1), USA (1)
182	12,1,5,2,3,1,20	1126	USA (1)
193	12,1,9,8,6,5,8	4210	Brazil (1), Cambodia (6), South Korea (1)
259	12,1,2,1,2,1,1	4214	China (6)
640	28,3,13,5,8,9,11	4201	Mexico (1), Spain (1)
761[b]	28,3,5,5,11,4,4	4211	NA
8	2,1,7,1,1,1,1	ATCC 12228 control	Canada (1), USA (1)

5 UN: Unknown, NA: Not applicable. a Countries and number of isolates according to the MLST database:
6 https://pubmlst.org/sepidermidis/ (until February 2022). b New ST found in this work.