African Journal of Microbiology Research Vol. 5(16), pp. 2266-2272, 18 August, 2011
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 ©2011 Academic Journals

Full Length Research Paper

Characterization of *Staphylococcus* spp strains isolated from hospital, community and environmental in Puebla city, Mexico

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Accepted 30 July, 2011

We investigated the prevalence of methicillin-resistant staphylococci (MRS) and evaluated the antimicrobial resistance patterns of 284 *Staphylococcus* strains isolated from two hospitals, as well as from community and the environment in Puebla City, Mexico. Isolates were identified by Gram-stain and biochemical reactions and antimicrobial susceptibility testing was performed by Kirby-Bauer and in some cases by determination of minimal inhibitory concentrations of antimicrobial agents. Result showed that from 284 strains studied, 32% (90 strains) were multidrug-resistant, of which 82% (74 strains) were methicillin-resistant. From 154 *Staphylococcus aureus* isolates tested, 41 strains showed methicillin-resistance (27%) and 84 of 130 coagulase-negative staphylococci showed methicillin-resistance (65%). The *mec*A gene was detected in 38 of 43 oxacillin-resistant staphylococci tested (14/14 *S. aureus* and 24/29 coagulase negative staphylococci). Nevertheless, this gene was also identified in 14 *S. aureus* that exhibited oxacillin and cefoxitin susceptibility. The percentages of resistance detected among *S. aureus* and coagulase negative staphylococcal isolates were: penicillin (79 and 67%, respectively), oxacillin (27 and 65%), erythromycin (18 and 36%), tetracycline (6 and 24%), gentamicin (71 and 45%), and trimethoprim sulfamethoxazole (19 and 37%). The β-lactamase production was positive in more than 80% of isolates. These results show the presence of multiresistant strains in these three sources, which supports the control measures taken by health authorities with respect to avoiding the misuse and abuse of antibiotics.

Key words: *Staphylococcus*, methicillin resistance *Staphylococcus aureus* (MRSA), methicillin resistance coagulase-negative staphylococci (MRCoNS), emerging infections.

INTRODUCTION

In the 60's, *Staphylococcus aureus* was identified as the main cause of nosocomial infections in the world (Klimek et al., 1976; Crossley et al., 1979; Doebbeling, 1995). At present, the methicillin-resistant *Staphylococcus* (MRS) and multiresistant *Staphylococcus* are the leading cause of infections in hospitals and community (Schaberg et al., 1991; Ponce de León, 1996; Diekema et al., 2000; Wu et

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al., 2006; Martins et al., 2007; David and Daum, 2010). Resistance to methicillin is due to the presence of Staphylococal Chromosomal Cassette *mec* (SCC*mec*) which in addition to carrying *mec*A gene that encodes a penicillin-binding protein (PBP2a) (Hartman and Tomasz, 1984; De Lencastre et al., 1994; Pinho et al., 2001), also can contains genes of resistance for non-beta-lactam agents, causing multidrug resistant strains (Katayama et al., 2000; Hiramatsu et al., 2001).

It is reported that *S. aureus* is the causal agent of more than 20% of bacteremias in USA, Canada and Latin America (Pfaller et al., 1998; 1999; Diekema et al., 2000; Wisplinghoff et al., 2003; Chen et al., 2010), skin infections and soft tissue (Gales et al., 2009; Doern et al., 1999) and pneumonia (Sader et al., 1998; Gales et al., 2009). Coagulase-negative staphylococci (CoNS) are the third most common type of isolates in bacteremias (Schaberg et al., 1991; Edmond et al., 1999; Diekema et al., 2001; Wisplinghoff et al., 2003; Chen et al., 2010, Rosa et al., 2009). In the environment, staphylococci are the most frequently isolated bacteria in both open and closed areas (Górny et al., 1999; Górny and Dutkiewicz, 2002; Tsai and Macher, 2005; Eames et al., 2009; Tang, 2009).

The emergence of multidrug-resistant staphylococci, prompted a warning from the health centers and organizations, because epidemiological changes, clinical manifestations and their control can become a significant Public Health problem in both developed and developing countries. In Latin America, it was reported a prevalence of 40% of methicillin resistant *Staphylococcus aureus* (MRSA) and over 80% methicillin resistant coagulase negative *Staphylococcus* (MRCoNS) and most of these strains were multidrug resistant (Sader et al., 2009). The World Health Organization (WHO) has seen the emergence and spread of antimicrobial resistance as a priority issue and therefore since September 2001 established a global measure for the containment of antimicrobial resistance, which includes as a fundamental measure the surveillance of antimicrobial resistance caused among other factors by self-medication (WHO, 2002). In Mexico, a law was issued in which the guidelines to which the sale and dispensing of antibiotics as a preventive measure to misuse of these drugs would be subjected (Official Gazette, 2010).

The aim of this study was to determine the methicillin resistance and other associated resistances of staphylococci strains isolated from two general hospitals, the community and the environment of the city of Puebla, Mexico.

MATERIALS AND METHODS

Bacterial isolates

284 staphylococci were included in this study. One-hundred-and-seventeen strains were obtained in two hospitals of Puebla, México (Pediatric Hospital in 2003; University Hospital of Puebla City in 1998-2000) and they were collected from wound and articulation secretions, sepsis, pleural and cerebrospinal fluids, peritoneal dialysis fluids, catheter tips, post-mortem and throat swabs. One-hundred-and-twelve strains were obtained from community during 2002-2003 and they were collected from outpatients with clinically reported staph infections (vulvar swabs, throat, skin and conjunctiva) as well as from hospital personnel and community carriers by means of throat swabs. By other side, 55 strains from the environment were collected in 2004, through the Gravity Sedimentation Technique (Frankland and Hart, 1887); specifically, blood agar and mannitol salt agar plates, were exposed for a period of approximately 15 and 45 minutes respectively, at a height of between 1.5 and 2.0 m (Lighthart and Shaffer, 1995; De la Rosa and Ullán, 2002; Rosas et al., 2004). *Staphylococcus* strains were identified by standard methods, such as colonial morphology, Gram-stain, catalase, coagulase, clumping factor, urease, DNase, hemolysis on blood agar and bacitracin test (Finegold and Baron, 1996).

Antimicrobial susceptibility test and β-lactamase production

All obtained staphylococci were tested by Kirby-Bauer test (CLSI, 2008) for susceptibility to the following antimicrobials oxacillin (OX, (1 μg/disk), penicillin (PE, 10 U), erythromycin (E, 15 μg), tetracycline (TE, 30 μg), gentamicin (GE, 10 μg), vancomycin (VA, 30 μg) and trimethoprim sulfamethoxazole (SXT, 25 μg) (Sanofi Diagnostics Pasteur, SA, Mexico City, Mexico). Mueller-Hinton agar (Oxoid, Cambridge, England) supplemented with 4% NaCl was used in these assays. The susceptibility of cefoxitin (FOX, 30 μg/disk) (Difco Laboratories, Detroit, MI, USA) was checked in a group of *mec*A-positive strains (n=14) that appeared as oxacillin-susceptible. Additionally, a minimum inhibitory concentration (MIC) test of oxacillin using the agar dilution method with Steers Replicator was performed for 90 staphylococci isolated from different origins (30 from each source). The results of disk diffusion tests and MICs were interpreted according to CLSI criteria (CLSI, 2008).

The β-lactamase production was performed by Cefinase disks (Becton Dickinson) according to manufacturer's instructions.

*mec*A gene amplification by polymerase chain reaction (PCR)

Specific PCR of the *mec*A gene was performed in 90 staphylococci from different origins that were also analyzed by MIC. Because the content of A + T in *mec*A gene is high (70%) and in order to minimize the amplification of DNA regions not related to the gene (Ubukata et al., 1990; Ryffel et al., 1990; Unal et al., 1992), we chose two sets of primers combined in two reactions, the sense primer P1, 5'-(911) GGTCCCATTAACTCTGAAG (929)-3' and antisense P3, 5'-(1956) AGTTCTGCAGTACCGGATTTGC (1935)-3' (Petinaki et al., 2001) and a pair designed in this study, MMecAF sense primer, 5'-(539) TCCAGAATGCAGAAAGACC (558)-3'and antisense MMecAR, 5'-(1076) TGTATGTGCGATTGTATTGCTATT (1053)-3', giving rise to PCR products of 1046 and 538 bp respectively.

RESULTS

From 284 isolates studied, 154 of them were identified as *S. aureus* (54%), isolated mostly from hospital and community sources and 130 isolates (46%) were CoNS, mainly obtained from environment and hospital sources. Methicillin-resistance in this study was firstly checked by the study of the susceptibility to oxacillin. Table 1 shows

Table 1. Species distribution and methicillin resistance (MR) in *Staphylococcus* strains from three sources analyzed.

Origin (n isolates)	Studied isolates		[a] Methicillin-resistance		OXA/FOX-susceptible
	S. aureus	CoSCN	*S. aureus* (%)	CoSCN (%)	*mec*A-positive *S. aureus*
Hospital (117)	71	46	24 (34)	36 (78)	5
Community (112)	80	32	17 (21)	15 (47)	6
Environmental (55)	3	52	0	33 (63)	3
Total: 284	154 (54%)	130 (46%)	41 (27)	84 (65)	14

CoSCN: coagulase negative staphylococci; OXA: oxacillin; FOX: cefoxitin
[a] Isolates included were those that presented oxacillin resistance (by Kirby Bauer or MIC).

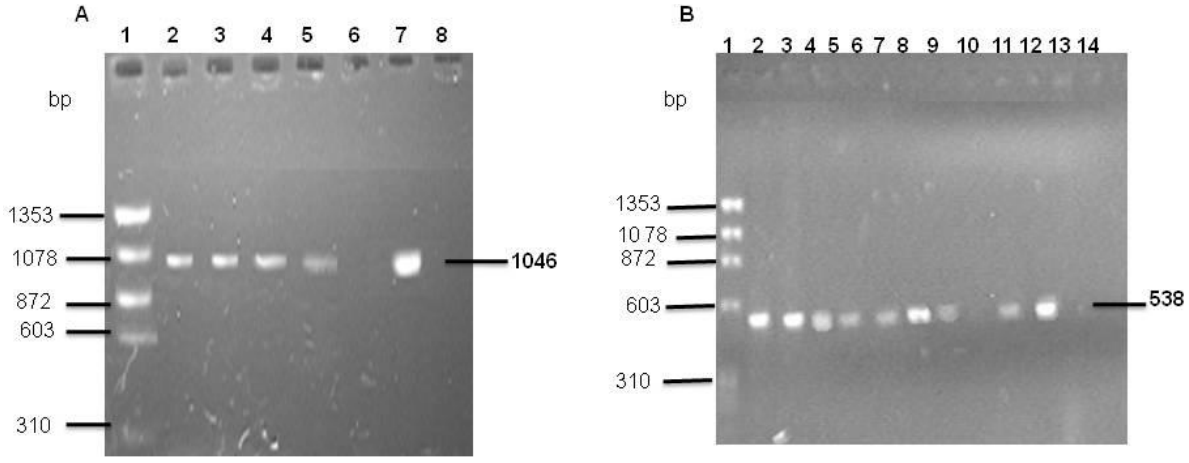

Figure 1. *mec*A gene detection by PCR, amplified in agarose gel and subjected to electrophoresis. A. P1F-P3R primers (Petinaki et al., 2001) to amplify 1046 bp. Line 1: Marker ϕX174; 2, 3-5, 7: *mec*A positive strains; 6: *mec*A negative strain and 8: ATCC 25923 (negative control). B. MmecAF-MmecAR primers to amplify 538 bp. Line 1: Marker ϕX174; 2, 3-8, 10 and 11: *mec*A positive strains; 9, 12 and 13: *mec*A negative strains and 14: ATCC 25923 (negative control).

the percentage of methicillin resistance among our isolates using this criterium. Forty-one *S. aureus* isolates of the 154 tested showed methicillin-resistance (27%), and isolates of hospital origin showed higher percentage of resistance than those of the community (34 and 21%, respectively). On the other hand, 84 of 130 coagulase-negative staphylococci showed methicillin-resistance (65%) and percentages were higher among hospital and environmental isolates (78 and 63%, respectively) (Table 1).

The presence of *mec*A gene was analyzed in a group of 90 staphylococci that included isolates of the three sources and also isolates with different oxacillin susceptibilities (Figure 1). The *mec*A gene was detected in 38 of 43 oxacillin-resistant staphylococci tested (14/14 *S. aureus* and 24/29 coagulase negative staphylococci, data not showed). Nevertheless, this gene was also identified in 14 *S. aureus* that exhibited oxacillin and cefoxitin susceptibility, observing a phenotypic-genotypic discrepancy (Table 1). From 90 selected strains and tested by β-lactamase production it was found that 100% hospital isolates, 83% community isolates and 93% environmental isolates were positive.

Figure 2 shows the percentages of resistance to different antimicrobial agents of *S. aureus* and coagulase-negative staphylococci. All strains tested were resistant to two or more agents and it was considered as multidrug-resistant strains those that were resistant to more than four antibiotics. From 284 strains studied, 32% (90 strains) were multidrug-resistant, of which 82% (74 strains) were methicillin-resistant. The percentages of resistance detected among *S. aureus* and coagulase negative staphylococcal isolates were: penicillin (79 and 67%, respectively), oxacillin (27 and 65%), erythromycin (18 and 36%), tetracycline (6 and 24%), gentamicin (71 and 45%), and trimethoprim sulfamethoxazole (19 and 37%).

DISCUSSION

As expected, the percentages of methicillin resistance detected among *S. aureus* and CoSCN in this study were higher in the hospital isolates in relation to those of the

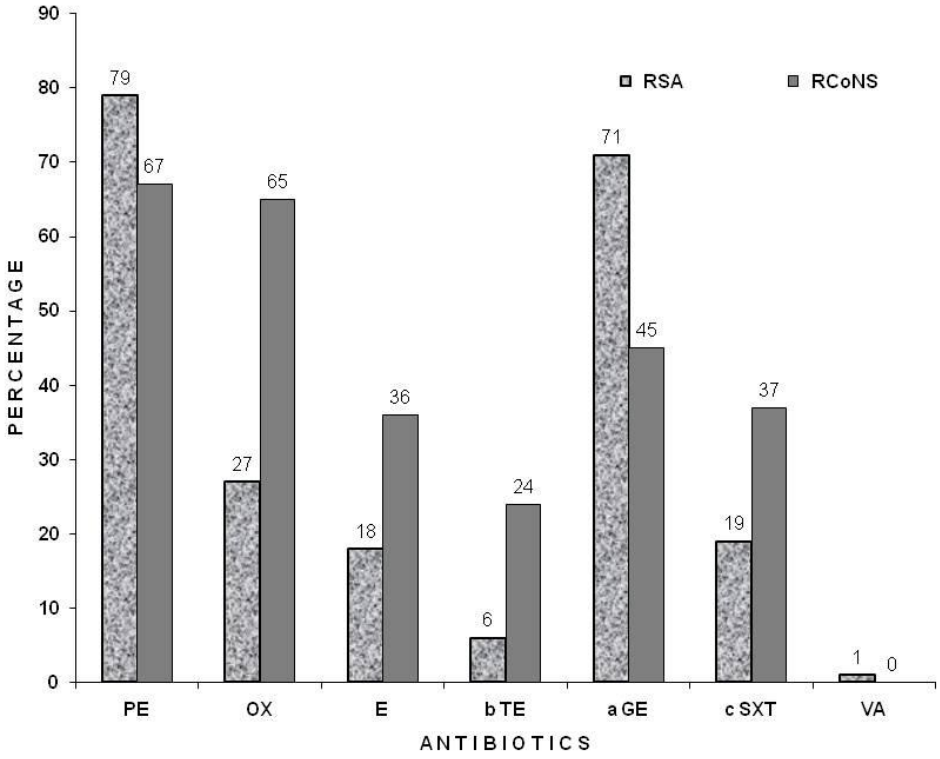

Figure 2. Resistance staphylococci strains isolated from Hospital, Community and Environmental by Kirby-Bauer test. PE: penicillin; OX: oxacillin; E: erythromycin; TE: tetracycline; GE: gentamicin; SXT: trimethoprim-sulfamethoxazole and VA: vancomycin. RSA, Resistant *Staphylococcus aureus* strains; RCoNS, Resistant Coagulase Negative *Staphylococcus* strains. Intermediate Resistance: [a] GE SA 8% and CoNS 6%; [b] TE CoNS 2% and [c] SXT 1%.

community. Hospital isolates presented high percentages of resistance (34 and 78% for *S. aureus* and CoSCN, respectively), what indicate that this type of resistant microorganisms can be a problem in the hospitals tested. These percentages are similar to other data previously published for Mexican hospitals and in other Latin American countries (Guzmán-Blanco et al., 2009).

The results of this study show that in our community *Staphylococcus* is a major concern for public health as a cause of emerging infections, due to the presence of multiresistant strains with ability to survive and spread. Among 284 strains collected in this study, the highest percentage was *S. aureus* isolated from community, which is consistent with that reported by the Centers for disease control and prevention (CDC) in USA (Kuehnert et al., 2006) and the Instituto Mexicano del Seguro Social (IMSS) in México (García-Contreras et al., 2000). The SCoN were isolated mostly from environmental sources, probably because they are found in greater numbers in skin and mucous membranes of both human animals as compared with *S. aureus* (Bischoff et al., 2004; 2007).

The resistance rates observed in this study agree with those reported in other regions (Diekema et al., 2001; Cuevas et al., 2004; Gales et al., 2009; Sader et al., 2009) and may be related to selective pressure to which bacteria are subjected in our community, because in the hospitals the committee epidemiological surveillance sets that the treatment of staphylococcal infections begins with β-lactam antibiotics, which in some cases, antimicrobial therapy of choice is subject to the standard antibiotic treatments available or unfinished treatments due to lack of resources of both health institutions and patients. In community, easy access to this type of antibiotics until 2010 year (Official Gazette, 2010) allowed the self-medication, resulting in inadequate doses or unfinished treatments (Dreser et al., 2008), favoring the spread of multiresistant strains in the community.

The GE resistance above 45%, consistent with that reported in 2005 by Public Health in Mexico (Benavides-Plascencia et al., 2005), where GE is among the seven antibiotics account for 80% of the observed resistance. In recent years, glycopeptides have been the mainstay of treatment of infections due to MRS. However, the isolation of CoNS with decreased susceptibility (Schwalbe et al., 1987), the report heteroresistant *S. aureus* strains (Hiramatsu et al., 1997) and the presence of strains resistant to VA (CDC, 2002; Palazzo et al., 2005; Tiwari and Sen, 2006), led that the treatment options for infections by MRS are committed and required of new antimicrobial agents (Appelbaum, 2006; Menezes et al.,

2008). In this paper, strains with intermediate susceptibility to VA were not identified however; the results emphasize the need for continuous monitoring of the levels of MIC to this antibiotic in the MRS, since these results alert of the risks and implications of the indiscriminate use of this drug. The E and SXT has been widely used in the treatment of staphylococcal infections (Huovinen et al., 1995; Schlegelová et al., 2002), however, clinical experience has shown that resistance to both drugs has developed worldwide (Huovinen, 2001; Gales et al., 2009; Sader et al., 2009). The TE is a relatively cheap antibiotic, has therefore been widely used in prophylaxis, treatment of infections and as animal growth promoter, so the selective pressure for its use, has led to resistant bacterial strains (Committee on Drug Use in Food Animals, 1999; Chopra and Roberts, 2001). The worldwide prevalence of resistance to TE in *S. aureus* is higher than CoNS (Diekema et al., 2001; Gales et al., 2009). However, in Spain was reported a resistance less than 5% in *S. aureus* and between 5% and 15% in CoNS (Pérez-Trallero and Iglesias, 2003). The TE resistance observed in this study is relatively low 6% for *S. aureus* and 24% for CoNS, which could be related to the TE is one of the lowest drug consumption in Mexico (Benavides-Plascencia et al., 2005) and is not considered a treatment of choice for serious staphylococcal infections (WHO, 1999). It is interesting intermediate susceptibility to GE, TE and SXT, while not reaching high levels, confirming the need for selection and rational use of these antibiotics.

The MRSA and MRCoNS *mec*A positive showed uniformity in the expression of resistance (Chambers, 1988), so that might be considered strains with homogeneous resistant (Weller, 1999). In the MSSA-*mec*A positive, the results confirmed with FOX could coincide with that reported by Hososaka et al. (2007), so the absence of the phenotypic expression of resistance in these strains, suggests new molecular targets that could be related to susceptibility to ME (Rohrer et al., 2003), or the presence of mutations in the *fem* genes, which are essential factors for ME resistance expression (Chambers, 1997), contributing to low levels of resistant to this antibiotic, without any alteration in the *mec*A gene (Giannouli et al., 2010). In MRCoNS-*mec*A negative, the expression of resistance to ME, could be related to different mechanisms for the production of PBP2a (McDougal and Thornsberry, 1986; Tomasz et al., 1989; Suzuki et al., 1993; Weller, 1999), resulting in the presence of extremely heteroresistant strains (Chambers, 1988). The MSCoNS-*mec*A positive strains reported as MR by test MIC, could be pre-MRCoNS (Hiramatsu, 1995), because mutations in regulatory genes of *mec*A would originate phenotypically methicillin resistant strains (Suzuki et al., 1993; Kobayashi et al., 1996; 1998).

In most of the strains tested the β-lactamase production was detected, suggesting that the regulatory genes *bla*R1 and *bla*I could also be related to the phenotypic expression of resistance (Cohen et al., 1972; Boyce et al., 1990; Hiramatsu et al., 1990; Ryffel et al., 1992; Hackbarth and Chambers, 1993; Hackbarth et al., 1994; Chambers, 1997; Rosato et al., 2003).

Finally, the results of this study confirm the presence of MRS strains, which are also multiresistant, whose mechanisms of resistance may not only be related to the interaction of the *mec* genes, but with much more complex regulatory mechanisms, so its worth conducting more detailed studies and further epidemiological surveillance of *Staphylococcus* strains isolated from different sources, to show the behavior over time of resistance in these strains, based on the measure introduced from August 2010 on the sale of antibiotics in pharmacies throughout Mexico through prescription (Official Gazette, 2010).

ACKNOWLEDGEMENT

Ana Marta de los Ángeles Lobo Sánchez was supported by a student scholarship from Consejo Nacional de Ciencia y Tecnología, México (CONACYT 174306).

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