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ORIGINAL PAPER

Antimicrobial susceptibility and invasive ability of *Staphylococcus aureus* isolates from mastitis from dairy backyard systems

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Abstract Fifteen (15) backyard farms were investigated to determine the antimicrobial susceptibility and invasion ability of *S. aureus* isolates from cows with subclinical mastitis in México. A total of 106 cows were sampled and 31 *S. aureus* isolates were recovered. *S. aureus* isolates were resistant to penicillin class antibiotics and susceptible to gentamicin and cetyltrimethylammonium bromide. STA9 and STA13 isolates were resistant to erythromycin (MIC > 25 μg/ml) and lincomycin (STA13, MIC > 25 μg/ml; STA9, MIC > 100 μg/ml). STA9 isolate harbors the *erm*(B) and *msr*(A) genes, whereas STA13 isolate harbors the *erm*(C) gene. STA9 and STA13 isolates contains the *lnu*(A) gene. Only 5 isolates (STA11, STA13, STA14, STA15 and STA21) were able to internalize in bovine mammary epithelial cells. These results indicate that *S. aureus* isolates from dairy backyard farms showed differences in the antimicrobial susceptibility patterns and invasion ability in bovine mammary epithelial cells. This kind of evaluations should be performed in different dairy regions, since resistance patterns and isolate diversity vary on a per-region basis.

Keywords Antimicrobial resistance · Dairy backyard farms · Invasion in epithelial cells · Mastitis · *Staphylococcus aureus*

Abbreviations

bMEC	Bovine mammary epithelial cells
MLS	Macrolides, lincosamide, and streptogramin antibiotics
QAC	Quaternary ammonium compounds
CTAB	Cetyltrimethylammonium bromide

Introduction

Bovine mastitis is a major disease affecting the dairy industry that results in economic loses and decreased animal health (Ruegg 2003). This disease is characterized by an inflammatory response of the mammary tissue. The predominantly contagious pathogens responsible for clinical and subclinical infections in lactating cows are the Gram positive bacteria *Staphylococcus aureus* (Kerro-Dego et al. 2002). Some bacteria responsible of mastitis have the ability to invade the mammary epithelium and the limited success of antibiotic therapy may be due to the ability of *S. aureus* to invade and survive within the cell (Buzzola et al. 2007). In addition, bacterial isolates associated with mastitis have different capacity to

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invade isolated bovine mammary epithelial cells (bMEC) (Wanasinghe 1981; Watts 1988; Yancey 1999; Anaya-López et al. 2006).

Antimicrobial therapy is a valuable tool for controlling mastitis and several studies regarding to susceptibility patterns of different mastitis pathogens have been reported elsewhere (De Oliveira et al. 2000; Anderson et al. 2006; Srinivasan et al. 2007). Different studies have demonstrated an increasing resistance frequency among mastitis pathogens (Myllys et al. 1998) or a growing susceptibility (Erskine et al. 2002), whereas others reporting no change (Makovec and Ruegg 2003). In the antimicrobial therapy of mastitis, macrolides, lincosamide, and streptogramin (MLS) antibiotics are used in the treatment of staphylococcal infections (Lüthje and Schwarz 2006). As a consequence, resistance to MLS antibiotics is prevalent among *S. aureus* and coagulase negative staphylococci (CoNS) and a large number of genes mediating resistance by different mechanisms have been identified (Khan et al. 2000; Lüthje et al. 2007; Lüthje and Schwarz 2007).

In México, milk production from dairy backyard systems contribute for the 35% of the national production, however these systems have been poorly studied. In contrast to the intensive systems, the backyard systems are confined to small spaces mainly in the house's backyards, the livestock does not have a high genetic quality and the practices of genetic improvement or preventive medicine are scarce (Méndez y Cazarín et al. 2000). Conversely, only limited information is available about the antimicrobial susceptibility and invasive ability of bacteria isolated from bovine maintained in dairy backyard systems (López-Meza et al. 2006). The aim of the present study was to evaluate the antimicrobial resistance and invasive ability of *S. aureus* isolates associated with mastitis from dairy backyard systems in Morelia, Michoacán, México.

Materials and methods

Staphylococcus aureus isolates

A total of 106 cows were analyzed and 31 *S. aureus* isolates were obtained from raw milk composite samples of cows with subclinical mastitis (determined by California mastitis test) maintained in 15 backyard farms in Morelia, Michoacán, México (Table 1). Isolates were collected from March 2004 to January 2005. Aliquots (200 µl) from milk samples were collected aseptically and distributed on Blood agar plates (Bioxon, México), then were incubated at 37°C for 24 h. *Staphylococcus aureus* isolates were identified by standard biochemical tests. The isolates were further characterized by molecular analysis amplifying the gene *nuc* encoding staphylococcal thermostable nuclease (Table 2) (Brakstad et al. 1992). The American Type Culture Collection (ATCC) *S. aureus* subsp. *aureus* 27543 strain, isolated from a case of clinical mastitis was included as a positive control. Prior to invasion assays bacteria were grown at 37°C overnight in Luria-Bertani broth (LB, Bioxon, México) and colony forming units (CFU) were adjusted by measuring the optical density at 600 nm. Isolates were identified by the letters STA followed by an arabic number.

Antimicrobials tests

All *S. aureus* isolates were tested for antibiotic and quaternary ammonium compounds (QAC) susceptibility. Antibiotics susceptibility was determined by the disk diffusion method on Mueller-Hinton (MH) agar plates (Bioxon, México). The following disks were used (Gram positive multidisc, Bio-Rad, México): ampicillin, 10 µg; cephalotin, 30 µg; cefotaxime, 30 µg; ceftazidime, 30 µg; cefuroxime, 30 µg; dicloxacillin, 1 µg; erythromycin, 15 µg; gentamicin, 10 µg; pefloxacin, 5 µg; penicillin, 10 U; tetracycline, 30 µg; trimethoprim, 25 µg. Isolates were classified as susceptible, intermediate and resistant according to the manufacturer's instructions. Susceptibility to lincomycin and QAC was tested on MH agar plates containing different concentrations of lincomycin (1–20 µg/ml, Pharmacia) and cetyltrimethylammonium bromide (1–10 µg/ml, CTAB, Sigma). A MH agar plate without antimicrobials was used as a control for each isolate. Plates were incubated at 37°C for 24 h. Isolates with confluent or semiconfluent growth on MH agar containing lincomycin at 10 µg/ml or CTAB at 6 µg/ml were considered resistant. The minimum inhibitory concentrations (MIC) were determined for several antimicrobial agents by a broth dilution method. The first dilution with no visible growth was considered as MIC for each isolate.

Table 1 Antimicrobial resistance patterns and invasive ability into bMEC of *Staphylococcus aureus* isolates associated with mastitis used in this study

Isolate	Internalized bacteria[a] (%)	Antimicrobial resistance patterns
STA1	–	AMP, CAZ, DX, PEN, CEP, CTX, CXM, SXT
STA2	–	AMP, CAZ, DX, PEN, CEP, CXM
STA3	–	AMP, CAZ, DX, PEN, CTX, CXM, SXT
STA4	–	AMP, CAZ, DX, PEN
STA5	–	AMP, CAZ, DX, PEN
STA6	–	AMP, CAZ, DX, PEN
STA7	–	AMP, CAZ, DX, PEN
STA8	–	AMP, CAZ, DX, PEN
STA9	–	AMP, CAZ, DX, PEN, CEP, CTX, CXM, LIN, E
STA10	–	AMP, CAZ, DX, PEN, PEF
STA11	62	AMP, CAZ, DX, PEN, CEP, CTX, CXM
STA12	–	AMP, CAZ, DX, PEN, PEF, TET
STA13	20	AMP, CAZ, DX, PEN, CEP, CTX, CXM, LIN, E
STA14	59	AMP, CAZ, DX, PEN, CEP, CTX, CXM
STA15	80	AMP, CAZ, DX, PEN, CEP, CTX, CXM
STA16	–	AMP, CAZ, DX, PEN, CEP, CTX, CXM
STA17	–	AMP, CAZ, DX, PEN
STA18	–	AMP, CAZ, DX, PEN, CTX, CXM
STA19	–	AMP, CAZ, DX, PEN, CEP, CTX, CXM
STA20	–	AMP, CAZ, DX, PEN
STA21	38	AMP, CAZ, DX, PEN, CEP, CTX, CXM
STA22	–	AMP, CAZ, DX, PEN
STA23	–	AMP, CAZ, DX, PEN
STA24	–	AMP, CAZ, DX, PEN
STA25	–	AMP, CAZ, DX, PEN
STA26	–	AMP, CAZ, DX, PEN
STA27	–	AMP, CAZ, DX, PEN
STA28	–	AMP, CAZ, DX, PEN, PEF
STA29	–	AMP, CAZ, DX, PEN
STA30	–	AMP, CAZ, DX, PEN, CXM
STA31	–	AMP, CAZ, DX, PEN
ATCC 27543	100	AMP, PEN

[a] Percentage of invasion considering the invasive ability of ATCC 27543 strain as 100%.

– = Isolates with values of invasion < 9%. Average of triplicates of three independent experiments is presented.

AMP—ampicillin; CAZ—ceftazidime; DX—dicloxacillin; PEN—penicillin; CEP—cephalotin; CTX—cefotaxime; CXM—cefuroxime; E—erythromycin; PEF—pefloxacin; TET—tetracycline; SXT—trimethoprim; LIN—lincomycin

PCR amplification of resistance genes

Genomic DNA was extracted from *S. aureus* isolates as reported previously and used as a template for amplification (Pospiech and Neumann 1995). Primers (Invitrogen) and PCR conditions used in this study were those reported by Lina et al. (1999). The primers allow the identification of 6 determinants of resistance to macrolides (erythromycin) and lincosamides (lincomycin) (Table 2). PCR was carried out on a GeneAmp PCR System 2400 (Perkin-Elmer). DNA amplification of *gyrA* was used to test the quality of the DNA extraction and for the absence of PCR inhibitors. PCR products were analyzed by electrophoresis through 1% agarose gels (Invitrogen).

Cell cultures

The isolation of primary bovine mammary epithelial cells (bMEC) was performed from alveolar tissue of udders of lactating cows as described previously (Anaya-López et al. 2006). Cells from passages 2nd to 8th were cultured in Petri dishes (Corning-Costar) in growth medium composed by DMEM medium/

Table 2 PCR primers used in this study

Specificity		Sequence	Fragment size (bp)	Annealing temperature °C and cycle number	Reference
Erythromycin *erm*(A)	Forward	5′-GTTCAAGAACAATCAATACAGAG-3′	421	52/30	Lina et al. (1999)
	Reverse	5′-GGATCAGGAAAAGGACATTTTAC-3′			
Erythromycin *erm*(B)	Forward	5′-CCGTTTACGAAATTGGAACAGGTAAAGGGC-3′	359	55/30	Lina et al. (1999)
	Reverse	5′-GAATCGAGACTTGAGTGTGC-3′			
Erythromycin *erm*(C)	Forward	5′-GCTAATATTGTTTAAATCGTCAATTCC-3′	572	52/30	Lina et al. (1999)
	Reverse	5′-GGATCAGGAAAAGGACATTTTAC-3′			
Erythromycin *msr*(A)	Forward	5′-GGCACAATAAGAGTGTTTAAAGG-3′	940	50/25	Lina et al. (1999)
	Reverse	5′-AAGTTATATCATGAATAGATTGTCCTGTT-3′			
Erythromycin *msr*(B)	Forward	5′-TATGATATCCATAATAATTATCCAATC-3′	595	50/25	Lina et al. (1999)
	Reverse	5′-AAGTTATATCATGAATAGATTGTCCTGTT-3′			
Lincomycin *lnu*(A)	Forward	5′-GGTGGCTGGGGGGTAGATGTATTAACTGG-3′	323	57/30	Lina et al. (1999)
	Reverse	5′-GCTTCTTTTGAAATACATGGTATTTTTCGATC-3′			
gyrA	Forward	5′-AGTACATCGTCGTATACTATATGG-3′	280	55/30	Lina et al. (1999)
	Reverse	5′-ATCACGTAACAGTTCAAGTGTG-3′			
S. aureus nuc	Forward	5′-GACTATTATTGGTTGATCCACCTG-3′	218	54/25	Brakstad et al. (1992)
	Reverse	5′-GCCTTGACGAACTAAAGCTTCG-3′			

nutrient mixture F-12 Ham (DMEM/F-12K, Sigma) supplemented with 10% fetal calf serum (Equitech-Bio), 10 μg/ml insulin (Sigma), 5 μg/ml hydrocortisone (Sigma), 100 U/ml penicillin and streptomycin (100 μg/ml) and 1 μg/ml amphotericin B (Invitrogen). Cells were grown in an atmosphere of 5% CO_2 at 37°C.

Invasion assays of *Staphylococcus aureus* isolates in bMEC

Confluent monolayers of bMEC were plated on 24 well plates (Corning) and infected with $\sim 6 \times 10^6$ CFU/well from *S. aureus* isolates with a multiplicity of infection (MOI) of 30:1 bacteria/cell. The bacteria were incubated with bMEC during 2 hours in DMEM/F-12K medium without serum and antibiotics in 5% CO_2 at 37°C. After infection, bMEC monolayers were washed three times with phosphate buffer saline (PBS) and incubated in F12K medium without serum supplemented with 50 μg/ml gentamicin for 2 h at 37°C to eliminate extracellular bacteria. Subsequently, bMEC monolayers were washed three times with PBS, detached with trypsin-EDTA (Sigma) and lysed with 150 μl of sterile distilled water. bMEC lysates were diluted 100-fold, plated on LB agar for triplicate and incubated overnight at 37°C. The number of CFU was determined by the standard colony counting technique.

For the transmission electron microscopy analysis, cells were fixed in 3% glutaraldehyde in phosphate buffer. Suspension was pelleted, dehydrated in an ethanol series and embedded in epoxic resin containing propylene oxide (1:1) for one day, and finally placed in resin, which polymerized in an oven at 60°C for 36 h. Sections were cut using an ultramicrotome, contrasted with uranyl acetate and lead citrate for observation with a JEOL-1010 transmission electron microscope operating at 80 kV.

Results

Collect of *Staphylococcus aureus* isolates from backyard systems and evaluation of antimicrobial susceptibility

In a regional program for the study of mastitis, 106 cows from 15 backyard systems were analyzed during the period of March 2004 to January 2005. According to California mastitis test, 29% of cows analyzed showed subclinical mastitis (31 cows). Samples of raw milk from these cows were collected and used to obtain *S. aureus* isolates. Thirty one *S. aureus* isolates were identified according to growth and biochemical properties (catalase, coagulase, gelatinase, and mannitol fermentation). The identification of the isolates was confirmed by PCR amplifying the gene *nuc* encoding staphylococcal thermostable nuclease. The environmental pathogens responsible of mastitis, *Escherichia coli* and *Klebsiella pneumoniae*, were also isolated but no were included in this study.

Antimicrobial susceptibility of standard *S. aureus* ATCC 27543 strain and 31 *S. aureus* isolates to 14 antimicrobials was evaluated (Table 1). The standard *S. aureus* ATCC 27543 strain showed resistance only to ampicillin and penicillin. In general, the *S. aureus* isolates from mastitis showed a variable susceptibility behavior towards the antibiotics tested. Isolates that were resistant to two or more antimicrobials belonging to different antimicrobial groups were considered as multidrug resistant. All *S. aureus* isolates were resistant to two or more antimicrobials. The 31 *S. aureus* isolates were resistant to ampicillin, ceftazidime, dicloxacillin and penicillin; many were resistant to cefuroxime (35%), cefotaxime (29%) and cephalotin (29%); and <10% were resistant to erythromycin, trimethoprim, pefloxacin, lincomycin and tetracycline (Table 1). All *S. aureus* isolates were susceptible to gentamicin (10 μg). Regarding to CTAB tests, all isolates showed growth in the presence of 1 μg/ml CTAB. Nevertheless, none of the isolates grew at concentrations >5 μg/ml CTAB. These results mean that all isolates are susceptible to CTAB.

We found that only STA9 and STA13 *S. aureus* isolates were resistant to erythromycin and lincomycin antibiotics (Table 1) and were selected to determined the MIC values. The STA9 isolate showed MIC values of >25 μg/ml to erythromycin and >100 μg/ml to lincomycin, whereas the STA13 isolate showed MIC values of >25 μg/ml to erythromycin and lincomycin. Further, STA9 and STA13 macrolides and lincosamide resistant isolates were investigated for the genetic basis of resistance. PCR analyses revealed that STA9 isolate harbors the *erm*(B) and *msr*(A) genes consistent with an erythromycin resistance phenotype. Also, the *lnu*(A) gene

was detected in the same isolate. In relation to STA13 isolate, the genes *erm*(C) and *lnu*(A) were detected. The other macrolides and lincosamide resistance genes were not detected in these isolates.

Invasion assays of *Staphylococcus aureus* isolates in bMEC

Bacterial invasion into epithelial cells is an important pathogenic mechanism for the establishment of the disease, due to this, the invasive ability of 31 *S. aureus* isolates and the standard *S. aureus* ATCC 27543 strain was evaluated in bMEC monolayers. According to CFU recovered, only 5 isolates (STA11, STA13, STA14, STA15 and STA21) were able to internalize into bMEC (Table 1) with values higher than 20% relative to the invasive ability of *S. aureus* ATCC 27543 strain. From these 5 isolates, the STA15 isolate showed the highest invasion ability (80%). The other isolates evaluated in this study have values <9% and were considered not able to penetrate bMEC cells. The internalization of *S. aureus* into bMEC was also demonstrated by transmission electron microscopy (Fig. 1).

Discussion

In this study, we assessed the antimicrobial resistance and invasion abilities of *S. aureus* isolates from cases of bovine mastitis in dairy backyard farms. This bacterium is one of the most frequently isolated pathogen causing clinical or subclinical mastitis (Kerro-Dego et al. 2002). In México the dairy backyard farms are very important for milk production, but the studies on antimicrobial resistance in bacteria associated to mastitis in these systems are poor (Méndez y Cazarín et al. 2000). Antimicrobial susceptibility evaluation for mastitis pathogens is complicated by the lack of interpretive criteria specific for most mastitis antimicrobials, and frequently the interpretation of antimicrobial susceptibility data uses the criteria applied with human data (De Oliveira et al. 2000). In this study, we detected a high level of resistance to penicillin class antibiotics, the 31 *S. aureus* isolates analyzed showed resistance to ampicillin, ceftazidime, dicloxacillin and penicillin (Table 1). In this region of study, penicillin class antibiotics have been frequently used for the control of mastitis, and exert a high selection pressure on the bacteria resulting in the development of resistance. Nevertheless, all isolates showed high sensitivity levels to gentamicin, and in less proportion to erythromycin and lincomycin. On the other hand, the dairy industry commonly uses quaternary ammonium-based detergents, such as CTAB and benzalkonium chloride, to clean and wash dairy equipment. As consequence, *S. aureus* isolates have developed resistance to such detergents (Bjorland et al. 2001). All 31 *S. aureus* isolates evaluated were susceptible to CTAB at concentrations >5 μg/ml detergent. The milk samples were from backyard farms where the application of disinfectant elaborated with QAC's is not a common practice. Thus, the absence of a selection pressure could explain the lack of resistance to CTAB.

A detailed evaluation of susceptibility to antibiotics of different classes is important in order to obtain basic information leading to the identification and characterization of the gene or gene pools that confer resistance to one particular antibiotic class. MLS antibiotics are widely used in the treatment of staphylococcal infections and resistance to antibiotics is prevalent among staphylococci (Lina et al. 1999; Lüthje and Schwarz 2006). We found two isolates (STA9 and STA13) with resistance to MLS erythromycin and lincomycin antibiotics. PCR analysis showed that isolate STA9 harbors the *erm*(B) and *msr*(A) erythromycin resistance determinant whereas the isolate STA13 only has the *erm*(C) gene. *erm*(B) and *erm*(C) genes confer resistance to MLS type B (MLS_B) by target site alteration of the ribosome,

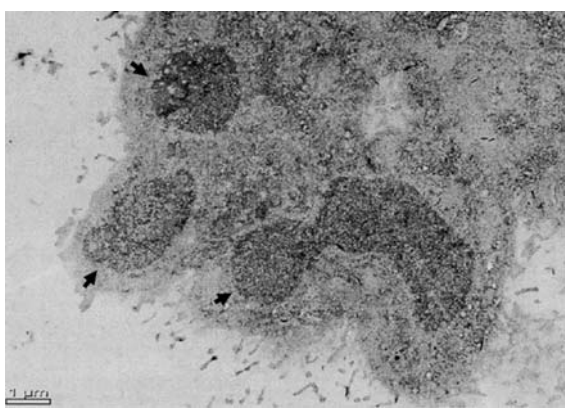

Fig. 1 Transmission electron micrograph of bMEC cell infected with *Staphylococcus aureus* ATCC 27543 strain. The bacteria are indicated by arrowheads. Bar = 1 μm

while *msr*(A) gene confers the MS phenotype (inducible resistance to macrolides and streptogramin type B after induction with erythromycin) by efflux (Roberts et al. 1999). In agreement, these gene determinants have been reported in staphylococci from bovine subclinical mastitis in Germany (Lüthje and Schwarz 2006). Likewise, both isolates have the *lnu*(A) genes which confer resistance to lincosamides. In this trend, we previously have reported the complete sequence of the *lnu*(A)-carrying plasmid pBMSa1 of *S. aureus* isolate from bovine mastitis collected in an extensive farm, responsible for lincomycin resistance (Loeza-Lara et al. 2004). In addition, recently several *lnu*(A)-carrying plasmids of staphylococci isolates from bovine mastitis have been characterized (Lüthje et al. 2007).

Although *S. aureus* is the most frequently isolated pathogen causing clinical or subclinical mastitis worldwide, in vitro studies have shown the invasion ability of only a few isolates into bovine mammary epithelial cells (Almeida et al. 1996; Hensen et al. 2000; Anaya-López et al. 2006). We found that 5 isolates were able to internalize into bMEC cells (Table 1). This invasion was corroborated by transmission electron microscopy (Fig. 1). In different countries only a limited number of isolates of *S. aureus* are responsible for most of the cases of bovine mastitis, which could be related to differences in their invasion or adhesion capability (Kerro-Dego et al. 2002). Even so, the pathogenesis of *S. aureus* infection is very complex, it has been demonstrated that *S. aureus* isolates belonging to different *agr* groups showing different abilities to invade MAC-T cells (Buzzola et al. 2007). However, the isolates used in our study need further molecular characterization in order to explain the differences detected in the degrees of internalization into bMEC cells.

In conclusion, antimicrobial resistance evaluation could be a usual practice in dairy backyard farms management. Additionally, the characterization of intracellular invasion of bacteria associated to bovine mastitis could be important for the understanding of this disease. This kind of evaluations should be performed in different dairy regions, since resistance patterns and isolate diversity vary on a per-region basis.

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