Methicillin-resistant *Staphylococcus aureus* (MRSA) Isolated from Colombian Foods

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doi:10.5618/bio.2012.v2.n1.6 || Received: 29-05-2012, Accepted: 12-07-2012, Available online: 16-07-2012

Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) was identified as a nosocomial pathogen in the 1960s and it is associated with serious community-acquired and nosocomial diseases, particularly in immuno-compromised patients. *S. aureus* also causes food poisoning and is the most important food-borne disease in Latin America. In Colombia, the Community acquired (CA-MRSA) increased by 4.4% between 2001 and 2006. However, the transmission of MRSA through food has not been well researched; its prevalence is unknown as is the risk of consumer exposure. It is important to determine the role of food in transmission of antibiotic-resistant bacteria, taking into account that the prevalence of MRSA varies according to geographical location. The aim of this study was to determine the prevalence of MRSA in Colombian foods and to typify isolated strains by detecting antimicrobial resistance, clonal groups and *Toxin A* gene. Resistance of 149 *S. aureus* strains isolated from food was determined by disk diffusion with oxacillin (1µg) and cefoxitin (30µg), Antimicrobial Susceptibility Test (AST) and *mecA* gene amplification. Positive strains were genotyped using pulsed field electrophoresis (PFGE). 5 (3.35%) of the 149 strains resistant to oxacillin and cefoxitin, were positive to *mecA* gene, indicating the presence of the SCC cassette. The electrophoretic pattern obtained by PFGE for these strains revealed that 4 (80%) of the 5 strains belong to the Chilean clone, with 100% genetic similarity; this clone has been associated with 65% of infections associated with health care. Furthermore, none of the 5 strains were positive to *Toxin A* gene. This is the first evidence of the presence of MRSA in food in Colombia. It is

necessary to continue this research by extending the sampling and collecting enough information to carry out risk analysis of the danger.

Keywords: Methicillin-resistant; *Staphylococcus aureus*; PCR; Antibiotic resistant.

1. Introduction

Staphylococcus aureus is considered the third most important cause of disease in the world among reported foodborne illnesses [1]. In the early 1960s, the first strain *Staphylococcus aureus* methicillin-resistant (MRSA) was described in the United Kingdom (UK), immediately after the introduction of methicillin into clinical practice [2]. This microorganism has emerged as a major public health concern worldwide because of its differential ability to spread and cause hospital-based and community outbreaks [3].

In the United States, during 1992–2003, the number of health care associated infections due to MRSA increased from 35.9% to 64.4% and in UK, death certificates increased by 39% [4]. In Colombia, the CA-MRSA (community-acquired, or community-associated *Staphylococcus aureus*) increased from 1% in 2001 to 5.4% in 2006 [5].

While environmental MRSA transmission has been investigated, transmission through food products has not received enough attention [1, 6]. However, Normmano et al established the presence of *S. aureus* strains that harboured the *mecA* gene isolated from food samples such as bovine milk, mozzarella cheese, and pecorino cheese [1]. Beside the available information reported, the current prevalence of resistant bacteria in food matrices and levels of MRSA consumer exposure risks remain unknown.

Nowadays the assessment of the activity of an antibiotic is crucial to the successful outcome of antimicrobial therapy; however the development of resistance both in human and animal bacterial pathogens has been associated with the extensive therapeutic use of antimicrobials or with their administration as growth promoters in meat product production [6, 7].

Recently, some investigators have suggested that bacteria may act as reservoirs of antibiotic resistant genes. Because of this, it is important to carry out studies to establish the role played by this microorganism in horizontal transfer of antibiotic resistant genes to intestinal microorganisms and food associated pathogenic bacteria [8]. These resistant bacteria may be transferred from meat product sources to humans by various means, such as the food supply (cattle, chickens, pigs and turkeys); this anti-microbial resistance results in human health consequences [9].

The major mechanism of resistance to β -lactam antibiotics is due to the acquisition of the *mecA* gene, which is located in the staphylococcal chromosome cassette, *mec* (SCC). The gene product of *mecA* is a penicillin-binding protein (PBP), designated PBP2a [10-12] that can function as a transpeptidase [13].

Besides methicillin resistance, MRSA strains are important in safe food because they are able to produce a variety of extracellular products as coagulase Staphylococcal enterotoxins (SEs) such as the classic enterotoxins SEB, SEC, SED, SEE and SEA [14]. SEA and SED are the most common enterotoxins associated with food poisoning outbreaks [15].

In order to achieve the detection of resistance of *Staphylococcus aureus* strains, various techniques have been described, such as the employment of a cefoxitin 30 μ g disc, using semiconfluent inoculums and overnight incubation at 35°C, resulting in a sensitivity of 100% and a specificity of 99%. In this way, disc diffusion remains the method of choice for routine screening for methicillin resistance in microbiological laboratories and is complemented by the *mecA* detection by PCR [16].

On the other hand, one of the most widely used molecular typing methods for the study of local epidemiology of MRSA is pulsed field gel electrophoresis (PFGE). This method has been used to identify MRSA clones that have a particular ability to cause major outbreaks [17, 18].

S. aureus isolated from nutritional matrices such as meat and dairy products were tested for MRSA using the disk diffusion test. MRSA strains were also genotyped and amplified by PCR in order to confirm the presence of the *mecA* and *Toxin A* genes and detect clonal groups.

2. Materials and Methods

Samples of food such as meat products, vegetables, milk and milk derivatives were evaluated for contamination with MRSA. A total of 149 strains isolated from 2004 to 2010 within LEMA (Laboratorio de Ecología Microbiana y de Alimentos, Bogotá, Colombia) were analyzed.

Control strains. Methicillin-susceptible *S. aureus* (MSSA) ATCC 29213 and MRSA ATCC 33591 strains were used as positive controls in susceptibility test. MRSA USA 300 and Chilean clone NCTC 8325 were used as PFGE positive controls.

2.1. Bacterial isolation. For staphylococci isolation, 10 g/mL of each sample were transferred to flasks with 90 mL of Peptone Water and then plated onto Baird Parker Agar (Scharlau, Spain). The samples were incubated at $35 \pm 2^\circ\text{C}$ for 48 h [19, 20].

2.2. Antimicrobial susceptibility testing. Tests for Oxacillin and Cefoxitin resistance were carried out using the Kirby-Bauer disc diffusion method. McFarland 0.5 suspension was spotted onto Mueller-Hinton agar (MHA) (Becton Dickinson). The concentration of cefoxitin and oxacillin used was 30 μ g and 1 μ g (BBLTM, USA) respectively [21, 22].

2.3. Identification of *nuc* and *mecA* genes. In order to confirm *S. aureus* species and *mecA* presence, PCR were performed amplifying *nuc*, *mecA* and *16S rRNA* (internal control) genes with primers previously described [23-25]. The mixture reaction for *mecA* consisted of 5.0 μ l of template DNA, 0.2 μ M of each primer, 2.0mM MgCL₂, 160 μ M dNTPs, 1X buffer and 0.5U Taq Polymerase (Invitrogen) in a final volume of 25 μ L. The reaction mixtures were subjected to 95°C for 3 min and then 30 cycles of 95°C for 10 s, 55°C for 30 s and 72°C for 30 s, in an MJ Mini Thermocycler (Bio-Rad Laboratories, Inc. USA). PCR amplification products were separated on a 1.5% agarose gel and visualized under UV light by the addition of ethidium bromide using Quantity One software (Bio-Rad).

2.4. DNA Preparation, digestion, and PFGE. The preparation of the chromosomal DNA of MRSA isolates and digestion of their genomic DNA with SmaI (Promega) were performed as described by Cruz et al. [26]. Electrophoresis was performed with a CHEF-DR II system (Bio-Rad Laboratories, Hercules, CA) with the following conditions: block 1: run time 10 h, switch time 5–15 s and voltage 6 V/cm; and block 2: run time 13 h, switch time 15–60 s and voltage 6 V/cm. The gels were stained and photographed using Quantity One software (Bio-Rad). Cluster analysis of SmaI macrorestriction profiles was performed and analyzed using the Dice similarity coefficient with a PFGE band similarity software (GelComparII software; Bio-Rad

Laboratories, Hercules, CA), using a cut-off of 85% as the criterion for cluster formation.

2.5. Antimicrobial Susceptibility test (AST). AST was performed only on the 5 MRSA strains identified thanks to the previously described methodologies, using the Phoenix AST method according to previously described techniques [14, 27]. The Phoenix AST broth was briefly supplemented by one drop of Phoenix AST indicator. From the standardized ID microbial suspension, 25 mL was transferred to the AST broth. The broth was then poured into the AST side of the panel for specific detection of *Staphylococcus* penicillinase and extended-spectrum beta-lactamase (ESBL).

2.6. Identification of Toxin A gene. The PCR reactions were carried out using 240 ng of genomic DNA extracted from the *mecA* positive strains [28]. All the amplifications were carried out in a thermal cycler Biorad PCR System, with initial denaturation at 94° C for 4 min followed by 30 cycles of denaturation at 94° C for 2 min, primer annealing at 55° C for 2 min and extension at 55° C for 2 min, followed by a final extension at 72° C for 7 min [29-31]. The amplified PCR products were visualized using standard gel electrophoresis in a 2% agarose gel, stained with ethidium bromide (0.05 mg/ml; Sigma Aldrich, Milan, Italy).

3. Results

Methicillin resistant *S. aureus* isolation from food products was performed as a preliminary test for the detection of the incidence of this food borne pathogen in

Colombia. Table 1 summarizes the results obtained with the disk diffusion method, *mecA* and *Toxin A* gene amplification, and PFGE. 10 (6.71%) of 149 presumptive *S. aureus* strains analyzed, were oxacillin and cefoxitin resistant, using an interpretive zone diameter of R< 10 mm. to oxacillin and R<19mm to cefoxitin [32-34].

Of the 149 presumptive *S. aureus*, 5 (3.35%) harbored *nuc* and *mecA* gene (**Figure 1**), four of them were isolated from ground beef (STA294, STA295, STA296, STA 297) and one strain from milk cream (STA54C).

The genomic DNA typing showed patterns of at least 9 fragments of 36.0674 kb in total in 5 MRSA isolates (**Fig. 2**). 4 of the 5 MRSA isolates showed a distinguishable banding PFGE pattern that match with the Chilean clone, with a 100% of genetic similarity. One isolate had a unique pattern not related to control clones.

All isolates with a *mecA* amplification (5 strains) were resistant to cefazolin, clindamycin, gentamicin, mupirocin high level, teicoplanin, trimethoprim-sulfamethoxazole, vancomycin, oxacilin and cefoxitin. STA 294 and STA 295 isolated from beef were also resistant to amoxicillin-clavulanate, ampicillin, ciprofloxacin, erythromycin and levofloxacin.

In respect to the enterotoxin A, the absence of the target staphylococcal toxin A gene strains was determined by the PCR for all of the *mecA* positive strains (5 isolates) tested in this study.

Table 1. Characterization of *S. aureus* isolates by presence of the *mecA* gene, disk diffusion method of cefoxitin and oxacillin, PFGE and *Toxin A* gene amplification.

STRAIN	DATE OF ISOLATION	FOOD	Inhibition zone (mm)		<i>mecA</i> gene	Chilean Clone	Toxin A gene
			Cefoxitin	Oxacilin			
STA 010	Donation Universidad Javeriana	-	0mm	0mm	-	Not tested	Not tested
STA105	8/07/04	Milk cream	0mm	0mm	+	-	-
STA 54C	8/07/04	Milk cream	0mm	0mm	-	Not tested	Not tested
STA 108	19/07/04	Fruit salad	0mm	0mm	-	Not tested	Not tested
STA126	21/07/04	Fruit salad	0mm	0mm	-	Not tested	Not tested
STA112	19/07/04	Fruit salad	0mm	0mm	-	Not tested	Not tested
STA294	2/10	Ground beef	0mm	0mm	+	+	-
STA295	2/10	Ground beef	0mm	0mm	+	+	-
STA296	2/10	Ground beef	0mm	0mm	+	+	-
STA297	2/10	Ground beef	0mm	0mm	+	+	-

Fig. 1. Agarose gel electrophoresis for the detection of *mecA* gen .1: molecular weight marker (DNA Ladder 50 bp. BioRad). 2-3: Positive control Strains. 4: STA010. 5: STA108. 6: STA105. 7: 73SAUR. 8: 59SAUR. 9:STA294. 10: STA295. 11:STA296. 12: STA297. 13: STA54C. 14: PCR without DNA.

Fig. 2. PFGE for the detection of MRSA strains clonal groups 1: Positive control– USA300 Clone. 2: Positive control– Chilean Clone. 3-7: Food isolated strains STA294, STA295, STA296, STA297, STA105.

4. Discussion

Detection of the *mecA* gene is considered the gold standard for MRSA confirmation [35]. However, phenotypic methods like oxacillin and cefoxitin disc diffusion are now an accepted method for the detection of MRSA by many reference groups including CLSI [16,

36]. However there are difficulties associated with this method, as other species of *Staphylococcus* genus can test positive. Therefore it is still necessary to confirm the specie previously. The diameter of the inhibition area may be affected by the concentration of inoculums, MHA composition, temperature, and duration of incubation and occasionally some *mecA* positive strains are not detected due to the low expression of resistance [37].

In this study, results of the cefoxitin and oxacillin disc diffusion test are in concordance with the PCR for *mecA* gene. Even only half of the tested strains amplified *mecA* gene, the remaining strains did not amplify for *nuc* gene, indicating that they were not *S. aureus* and then they should not amplify for the staphylococcal chromosome cassette (SCC). This findings agrees with the statement by KB Anand in 2009 [38], who argued that the diffusion method is very suitable for the detection of MRSA, and the test can be an alternative to PCR for detection of MRSA in settings characterized by resource constraints.

In this survey of 149 strains, 5 (3.0%) were methicillin resistant (MRSA) percentage which is according to that stated by Normanno in 2007, who found that 3.75% of 160 *S. aureus* strains isolated from food were MRSA harboring the *mecA* gene [1]. In another study Lee [39, 40] found 15 (0.78%) of 1913 strains harboring the *mecA* gene. Most of the MRSA isolates found by the authors in this study, were from milk and cattle, which supports our findings with respect to MRSA isolation origin (**Table 1**). The source of food contamination is unclear since previous available reports are few and this is the first report about the presence of MRSA in Colombian foods.

The first molecular characterization of MRSA in Colombia was performed with clinical isolates recovered between 1996 and 1998; their results revealed that almost all of MRSA isolates belonged to a pediatric clone [5]. However, later studies observed that isolates with the characteristics of CA-MRSA strains increased in prevalence in Colombia between 2001 and 2006, belonging to the Chilean clone [26, 41]. Our findings suggest the presence of the same clone in food samples, showing MRSA's ability to disperse.

Suggested explanations for this phenomenon included the increased migration of human populations, combined with ineffective practices to control the spread of MRSA from infected or colonized patients [41]. Nevertheless, the prevalence of MRSA largely depends on the region and site of infection [42]. However, this study has demonstrated that MRSA has entered the food chain through the presence of MRSA in meat products

in Colombia.

The antibiotics profile showed multiple antibiotic-resistances in all MRSA strains, which is important given that *S. aureus* is a common cause of nosocomial infections and a frequent cause of community-acquired infections [32]. Furthermore, these infections are commonly treated using beta-lactam antibiotics, and *S. aureus* had developed marked resistance to this [32]. Contamination of food products should be traced in order to identify potential threats of MRSA acquisition by consumers.

According to Normanno [1], MRSA strains are able to synthesize different kinds of enterotoxins alone or in association, providing further evidence that MRSA may also be involved in food poisoning outbreaks.

The identification of staphylococcal *toxin A* gene in *S. aureus* strains by PCR offers a very specific, sensitive and inexpensive alternative to traditional immunological assays, which depend on adequate gene expression for reliability and sensitivity. The absence of the target staphylococcal *toxin A* gene strains was determined by the PCR for all of the *mecA* positive strains (5 isolates) tested in this study. However it does not mean that Staphylococcal toxins were absent, because it is necessary to evaluate them for all other known enterotoxin genes *seB* to *seI* [43].

These findings are important because they show the incidence of MRSA in Colombia and are the first to be shown in the country. It is necessary to continue this research by extending sampling and collecting enough information to carry out a risk analysis of this danger and for the epidemiological control of this pathogen.

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