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Methicillin resistant *Staphylococcus aureus* isolated from meat raw in Cartagena, Colombia

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Staphylococcus aureus resistente a meticilina aislado de productos cárnicos crudos en Cartagena, Colombia

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ABSTRACT

To determine the prevalence of Methicillin Resistant *Staphylococcus aureus* (MRSA) isolated in establishments that commercialize raw ground beef and pork chops in Cartagena- Colombia. 160 samples were analyzed through microbiological cultures in Baire Parcker agar, and it was determined the presence of mecA gen that codifies the methicillin resistance and the pvl that codifies the Panton- Valentine leukocidin toxin (PVL) by the multiplex PCR technique. The antibiotic susceptibility profile for MRSA strains was realized by automatized methods and for MSSA strains it was used Kirby Bauver. 66 samples were confirmed as *S. aureus* by PCR. The prevalence of MRSA was 7.5% and 33.8% of MSSA. The 66% of the strains were isolated from raw ground beef and the 34% of pork chop meat. The isolations presented about 2 – 12% of multi-resistance to the antibiotics used. The MRSA showed resistance to amoxicillin- clavulanate (57%), ampicillin-sulbactam and cefazolin (85%), erythromycin and clindamycin (7%), tetracycline (35%). The 10% of the isolated strains had the gen of PVL toxin and the 71% of those were identified in samples of raw pork meat and the 28% in raw ground beef. This study reports for the first time, how meat raw products commercialized in the city of Cartagena could build a dissemination source of MRSA carrier of PVL toxin that could generate a public health disease.

Key words:
MRSA
MSSA
PCR
Panton Valentine
Leukocidin toxin (PVL)
Meat products
Bacterial resistance

RESUMEN

Se determinó la prevalencia de *Staphylococcus aureus* resistente a Meticilina (SARM) aislado en expendios que comercializaban carne cruda molida de res y chuleta de cerdo en Cartagena - Colombia. Fueron analizadas 160 muestras a través de cultivo microbiológico en agar Baire Parcker y se determinó la presencia del gen *mecA* que codifica la resistencia a la meticilina y de *pvl* que codifica la toxina leucocidina de Panton-Valentine (PVL) mediante la técnica de PCR múltiple. El perfil de susceptibilidad antibiótica para las cepas SARM fue realizado por métodos automatizados y para cepas SASM se utilizó Kirby Bauver. 66 muestras fueron confirmadas como *S. aureus* por PCR, la prevalencia de SARM fue de 7.5% y 33.8% de SASM. El 66% de las cepas fueron aisladas de carne molida de res y 34% de carne de chuleta de cerdo. Los aislamientos presentaron entre 2 a 12% de multirresistencia a los antibióticos utilizados. Los SARM mostraron resistencia a amoxacilina-clavulonato (57%), ampicilina – sulbatam y cefazolina (85%), eritromicina y clindamicina (7%), tetraciclina (35%). El 10% de las cepas aisladas tenían el gen de la toxina PVL y el 71% de estos fue identificado en muestras de carne cruda de cerdo y el 28% en carne cruda molida de res. Este estudio reporta por primera vez, como productos cárnicos crudos comercializados en la ciudad de Cartagena pueden constituir una fuente de diseminación de cepas SARM portadoras de la toxina PVL, lo cual podría generar un problema de salud pública.

Palabras claves:
SARM
SASM
PCR
Toxina Panton Valentin
Leucocidina (PVL)
Productos cárnicos
Resistencia bacteriana

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Since its isolation in 1961 until now, methicillin resistant *Staphylococcus aureus* (MRSA) has been considerated one of the main pathogens healthcare-associated infections at hospitals, nevertheless, in the last two decades the scene of infections caused by this bacteria has been changing due to the emergency and dissemination of productive strains of community acquired infections, known as CA-MRSA (Bustos *et al*., 2006). This strains defer from the traditional hospitalarie ones, not only in their epidemiological behaviour but also in their susceptibility to antibiotics and virulence (Zetola *et al*., 2005). The CA-MRSA strains has been showed virulent features, and have caused serious outbreaks in gated communities as family, millitary groups, recluses, kindergartners and athletes (Ho *et al*., 2007). Most of these strains produce the toxin Panton-Valentine leukocidin (PVL), which has been associated with necrotizing pneumonia in all age groups; containing the chromosomal cassette SCCmec type IV or V, which confers methicillin resistance which is encoded in the *mecA* gene that is located in this cassette (McClure *et al*., 2006; Hiramatsu *et al*., 2001). This genetic vehicle can carry other genes as Tn554 gene that confers macrolid, clindamycin and streptogramins resistance, the pT181 gene, confers tetracycline resistance. The same SSCmec can carry virulence genes enterotoxin B and C and toxic shock toxin (Barbara-Wysockib *et al*., 2010; Zuo *et al*., 2008). Additionally, MRSA strains have the ability to produce intermittent colonizations (children, 10-40% and adults, 30%) being the most common site, the nasal cavity, which can lead to infections with severity varying degrees as infections of the skin and tissues through osteomyelitis and rapidly evolving and high mortality necrotizing pneumonia (Quiroga *et al.,* 2013).

Regarding to multi-resistant *Staphylococcus aureus* strains related with food industry it has been shown that these has predominantly colonized people who works in the production of animal origin food. It also has been reported high prevalence of *S. aureus* on pigs of different parts of the world (Smith and Pearson, 2010; Rijen *et al*., 2007). By the way, studies such as Gilchrist *et al*. (2007), have shown contamination of meat with multiresistant *S. aureus* to clinically important antibiotics as Ciprofloxacin, quinupristin / dalfopristin, clindamycin, erythromycin, oxacillin, and daptomycin. Also in a study made in Colombia, they found that the prevalence of resistance to erythromycin and clindamycin in *S. aureus*, was around 57 and 58%, respectively (Reyes *et al*., 2007). In the city of Cartagena they have been studies that report the presence of CA-MRSA in individuals with no clinical antecedents. Álvarez *et al*. (2006), in patients with nasal pathology treated in the Otolaryngology service at the Universitary Hospital of the Caribbean in Cartagena, reported a prevalence of *S. aureus* 22.8% and 5.26% for MRSA; another investigation by Bettin *et al*. (2012), reported that the 16% of the Medical students carry at least one of the MRSA strains in their nostrils for one or two years of their stay in clinics, where they perform their professional practices. The purpose of this study was to determine the prevalence of *S. aureus* CA-MRSA and antimicrobial susceptibility profile from samples of ground beef and raw pork meat sold in the city of Cartagena, in order to meet potential sources of spread of such pathogenic microorganisms.

MATERIALS AND METHODS

Obtaining the samples

An observational descriptive study was conducted in 40 establishments which commercialize beef and raw pork, distributed in three locations in the city of Cartagena: 19 (47.5%) in the localidad histórica y del Caribe (LHC); 16 (40%) in the localidad industrial de la bahía (LIB) and 5 (12.5%) in the localidad turística (LT), where the statistical criteria for the selection of the shops that marketed the two types of meat was: estimated variance of 0.19 according to preliminary investigation; a 5% of error and a minimum probability of 75%. The establishments (sampling units) were randomly selected and in each one dispensing samples between 100-300 g of ground beef (CM) and raw pork chop meat (CC) were taken. The sampling was conducted from July 2012 to May 2013, taking into account the guidelines set out in the Norma Técnica Colombiana NTC 4491-2 (2004).

Microbiological analysis

Once the samples were taken, these were labeled, refrigarated to 4 °C and transported to the science laboratory of food at the Universidad del Sinú-Seccional Cartagena. Microbiological cultures were performed for the count of *S. aureus* through the count in Baird Parcker® agar plate technique. The procedure consisted on performing three samples of serial dilutions (10^{-1}, 10^{-2} and 10^{-3}), and plated in duplicates, incubated it for 48 hours at 35 °C,

according to INVIMA (1998), and the NTC 4779 (2007). The sample reading was realized according to the NTC 1325 (2008) that establishes as an aceptable microbiological requirement for *S. aureus* 100-300 CFU g^{-1} of meat raw procesed, cool and/ or frozen products. Samples were the growment was not showed, were reported as <100 CFU g^{-1}. The specificity and sensibility controls of the culture medium realizated with the *S. aureus* sample ATCC 25923.

Molecular Identification

The genomic DNA in each isolation was reached according to the protocole described by Bettin *et al*. (2012). Were each presumptive isolation was cultured on a Plate Count® agar and incubated for 24 h at 37 °C. Five colonies were suspended in 0.5 mL of TE buffer and centrifugated at 13,000 rpm x 5 min, heated for 30 min and finally centrifuged at 13,000 rpm for 15 min. The supernatant that contains the bacterial DNA was stored at -20 °C until a subsequent amplification through the multiplex polimerase chain reaction (M-PCR).

Polymerase chain reaction (PCR) test

Every isolated MRSA were tipifyed using a multiple PCR essay according with the protocole described by (Zhang *et al*., 2005). The recolected DNA was subjected to a M-PCR to amplify the *mecA* gene (who determine the methicillin resistance), that amplifies a 147 pb fragment, the *nuc* gene of the specific *S.aureus* thermostable nuclease that amplifies a 300 pb fragment and the 437 pb of the *Luk*-PVL of the PVL toxin. The control strains were *S. aureus* ATCC 33591 (*mecA* +; *nuc* +; PVL -) and ATCC 25923 (*mecA* -; *nuc* +; PVL +) and water for negative control of the PCR assays. The DNA was amplified in a reaction volume of 25uL containing 12.5 uL of PCR mix (PCR master Mix; Promega), 0.2 uM of each primer and 5 uL of template DNA. The reaction was conducted in an Axigen® thermal cycler under the following conditions: an initial cycle of denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C 2 min with a final extension cycle at 72 °C for 10 min. All products were visualized on agarose gel 1.5% with ethidium bromide (0.5 ug mL^{-1}), using a UV transilluminator.

Antibiotic susceptibility profile. The method used to identify MRSA strains was the MicroScan automated system, Siemens®, using panels for dehydrated Gram possitive, supplied by Siemens. For the MSSA strains was used the diffusion method on Kirby Bauver disc. The MSSA strains were determined the following antimicrobials susceptibility: gentamicin 10 mg, 15 mg erythromycin, trimethoprim / sulfamethoxazole 23.75 mg and 1.25 mg, 2 mg clindamycin and rifampin 5mg, those antibiotics were supplied by DIFCO. For oxacillin resistance mediated by the *mecA* gene, was performed as a screening test the microdilution agar technique and a disk diffusion with 30 ng cefoxitin (DIFCO) in a Mueller-Hinton agar. The plates were incubated at 35 °C between 16 to 20 hours. Reading and interpretation was performed according to the guide lines of CLSI, (2013). The possitive *mecA* control strain was *S. aureus* ATCC 43300.

RESULTS AND DISCUSSION

Emergence of methicillin resistant *Staphylococcus aureus* strains in the community with different characteristics from MRSA hospital strains, has caused an alert among health centers and organizations dedicated to the study and control of this microorganism. CA-MRSA infections are an emerging problem in many parts of the world. The injury level yet known, the epidemiological change, clinical manifestations and control can become into a significant public health problem in the near future. Establishing health measures it's neccesary to control the re-emergence of this pathogen and, as possible, to eliminate it in time for preventing it to become a serious threat to the community. In total 160 samples were taken in 40 establishment that comercialized raw meat products, 80 samples of ground beef and 80 samples of pork chop meat. 47.5% (76/160) of the samples was taken in the LHC, 40% in the industrial bay place (LIB) and 12.5% in the tourist place (LT).

From the 160 samples analyzed, in 46% of the samples (75/160) were obtained presumptive counts os *S. aureus* greater than 100 CFU g^{-1} in 54% (85/160) no microbial growth was obtained, which is reported as counts <100 CFU g^{-1}. At the 75th samples counted > 100 CFU g^{-1} were subjected to the technique of polymerase chain reaction (PCR) to confirm the identification of the bacterial genus and in 66 samples (88%) the presence of *S. aureus* was confirmed and in 9 (12%) was not possible to determine the genus. From the 66 samples confirmed by PCR, 18% (12/66) were positive for the *mecA* gene indicating that they were MRSA strains, and 54 (82%) did not have the gene, confirming them as methicillin sensitive or MSSA strains. As for its origin, 66% were isolated from ground

beef meat and 34% pork chop, regardless if they were MRSA or MSSA. Regarding to it provinance, the 66% were isolated from ground beef and 34% from pork chop meat regardless if they were MSRA or MSSA.

From the 66 confirmed samples, 23 (35%) had acceptable microbiological counts of food quality (100-300 CFU g^{-1}) according to the Norma Técnica Colombiana NTC 1325 (2008); and 43 samples (65%) presented higher counts than 300 CFU g^{-1}. From the 12 possitive samples for MRAS, just 4 of them had acceptable quality counts, while 8 had counts above the accepted standard. The results of the isolated global prevalence of MRAS and MSSA are presented in the Table 1. Furthermore, in Figure 1 the results of PCR amplified products for the presence of *mecA* in positive samples are shown.

Table 1. Prevalence of Methicillin resistant *Staphylococcus aureus* (MRSA) and sensible (MSSA) in beef and pork samples

Count (FCU g^{-1})	Samples (n)	MRSA	MSSA	Other Genus
		(%)		
< 100	85*	0	0	0
100 – 300	32	4 (125)	19 (594)	9 (281)
> 300	43	8 (186)	35 (814)	0
Total	160	12 (75)	54 (338)	9 (56)

* Number of samples detected was not microbial growth.

Most animals can be colonized by *S. aureus*, various researches reports isolated MRSA strains from pigs, cows, chicken and others (De Neeling *et al*., 2007; Huijsdens *et al*., 2006), especially reported colonization cases by this strain in pigs, farmers and their families. In Netherland people who are in contact with pigs it's recognized as a risk factor for MRSA (Van Duijkeren *et al*., 2007), according to this, it has been suggested the relation to the emergence of MRSA strains in pigs and the antibiotic use in agriculture (Wulf and Voss, 2008). Results of this research reports the evidence of MRSA in meat products comercialized in the city of Cartagena, and the increase of this organism in relation to the virulence and pathogenicity, coinciding with other researches were the identification and development of new cases in different geographical locations has been confirmed by the implications on public health in both developed and developing countries (Lim *et al*., 2012; Deurenberg *et al*., 2006).

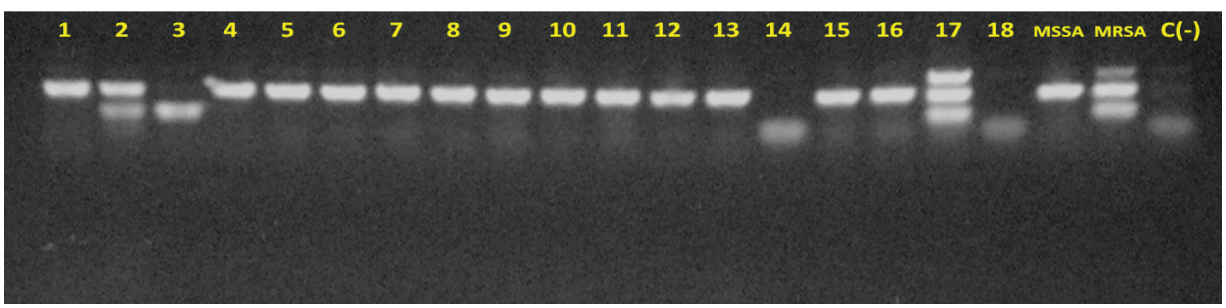

Figure 1. Electrophoresis in Agarose gel of the products of multiplex PCR, indicating possitive detection from the *nuc* gene (MSSA) and *mecA* (MRSA) in the analized strains. Lanes 1, 4-13, 15 and 16 indicate confirmation MSSA strains. Lanes 3, 14 and 18 indicate that there was not *nuc* detection, they do not belong to the Staphylococcus genus. Lanes 2 and 17 indicate the presence of MRSA and additionally to the presence of PVL gene in strain of the lane 17. C (-) negative control.

For the distribution of MRSA strains it was observed that there was the presence of these strains in the three locations, being LIB and LT the localities that contributed with four isolates each one, and the localidad del caribe

norte contributed with two. There were 10 establishments in total which the presence of the *mecA* gene in the analyzed samples was there. The presence of the PVL gene was found in 7 (10.6%) of the 66 strains confirmed as *S. aureus* by PCR, of which six samples, two of ground beef and 4 pork meat were identified as MRSA and only one of the pork meat as MSSA. This gene was isolated in the (71%) of the pork chop meat samples and in the (28%) of the ground beef samples, one of the pork isolated strains was identified as MSSA. Studies show the prevalence of MRSA in ground raw meat (Fontes *et al*., 2013; Boer *et al*., 2009; Hanson *et al*., 2011) in agreement with our results. The presence of this strain in food may be due to the contamination of the slaughterhouse ducts or by the deficient hygiene practice of the operator, inasmuch as nasal area is considered the primary colonized site by *S. aureus,* who is also founded in the intestinal tract (Bhalla *et al*., 2007).

Two of the samples presented the encoding gene for the PVL toxin, they were isolated from meat samples with colony counts that were between the reference parameters: 100-300 CFU g^{-1} (Table 2) which is considered suitable for human consumption, five of the samples with the presence of this gene were rejected by showing higher counts than 300 CFU g^{-1}, according to the Norma Técnica Colombiana NTC 1325 (2008). The establishments were the toxin genes were found were distributed in the three localities, and corresponds to two establishments per locality. Presence of MRSA strains that carriers PVL toxin in establishments in the three localities in the city Cartagena constitutes a risk to the population because it could be presented pathologies associated to this toxin that has the ability of destroying white blood cells and also necrotizing injuries in lungs and soft tissues (Rossney *et al*., 2007). Because of the invasive and virulent ability of this toxin, it has reached a great importance and concern in society, which currently conducted numerous studies in different countries about the isolation of MRSA's capable of producing the toxin (Witte *et al*., 2005). According to this Hanson *et al.* (2011) reported two isolated strains from pork meat, with a global prevalence of 1.2%; instead just one isolated MRSA was possitive to the PVL gene. A recent study in Japan identified MSSA t034 in pigs (Asai *et al*., 2012). Another study in China described T189, associated with ST188, as one of the more common clones responsible for bacteremia (Yu *et al*., 2012). Therefore, food handlers carrying *S. aureus* T189 may present higher risk of infection and food transmission.

Table 2. Colony count (CFU g^{-1}) vs presence of the encoding gene for the PVL toxin.

Code Samples	Type of sample	count FCU g^{-1}	present / absent MRSA	gen PVL
5CM1	Beef	1500	positive	positive
27CM1		600	positive	positive
5CC1	Pork chop	500	positive	positive
27CC1		600	positive	positive
31CC1		200	positive	positive
55CC1		1700	positive	positive
23CC2		200	negative	positive

Figure 2 shows the percentage results of multiresistant strains of *S. aureus* isolated from meat products. Antibiotic multidrug-resistance were observed in the 66 isolated strains. One of the isolated strain showed resistance to 8 antibiotics (Amoxicillin-clavulanate, erythromycin, clindamycin, tetracyclines and cefazolin, penicillin, rifampin and ampicillin sulbatam), but even so it was identified as MSSA. MRSA strains were resistant to the following antibiotics: 57% amoxacilln-clavulonate, ampicillin - sulbactam and cefazolin, 85% penicillin, 7% erythromycin and clindamycin, 35% to tetracycline.

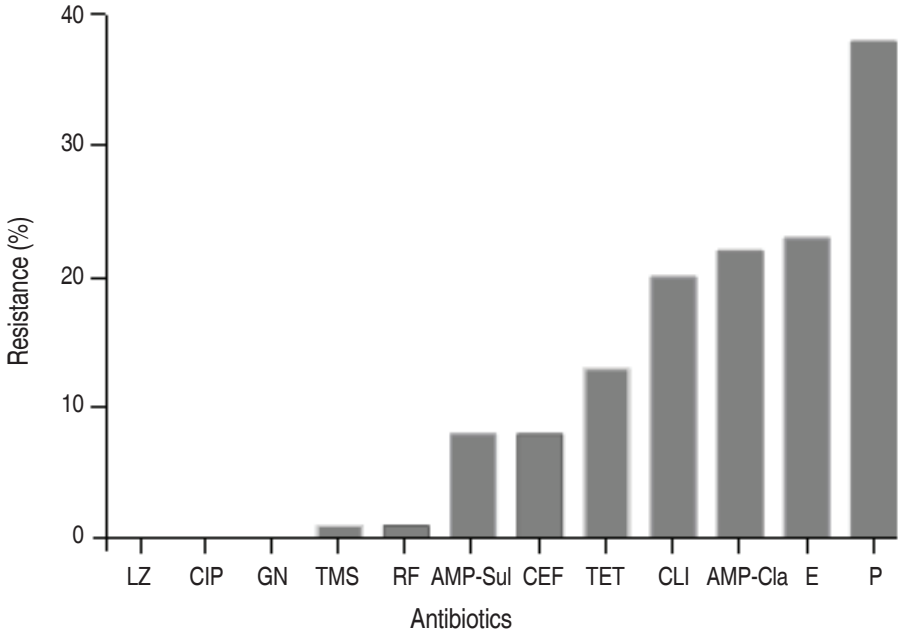

Figure 2. Strains percentage of multirresistant *S. aureus* isolated from ground meat products (n=66). Antibiotics: LZ: linezolid; CIP: ciprofloxacin, GN: gentamicin, TMS: Timetrophrim- sulfametoxazol, RF: rifampicin, AMP-Sul: ampicillin-sulbactam, CEF: cefazoline, TET. Tetraciclyn, CLI: clindamycin, AMP-Cla: ampicillin- Clavulanic acid, E: eritromycin, P: penicillin.

Regarding the antibiotic susceptibility of *S. aureus* isolated in food, the study of Gilchrist *et al*. (2007) where 5136 samples of meat and poultry were analyzed, founding that 47% of the samples were contaminated with *S. aureus*, which 52% of isolates showed multidrug resistance to antibiotics, which allows to state that the widespread use of antimicrobials in food production of animal origin, could be one of the causes of the emergence of multidrug resistance. A study by Ho *et al.* (2014) in food handlers made in six food companies, showed that people exposed to raw meat have higher risk of being colonized with *S. aureus* strains, it was also reported strains resistant to tetracycline (20%) and erythromycin (16%). So it is possible that multidrug-resistant strains come from food handlers.

Another study conducted in five cities of USA, founding in beef and poultry, multiple resistance to clinically important antibiotics like ciprofloxacin, quinupristin / dalfopristin, clindamycin, erythromycin, oxacillin, and daptomycin (Marty *et al*., 2012). This study is consistent with our results, as it was found highly resistant to antibiotics commonly used to treat infections caused by this bacterium. MRSA has apparently responded well to therapies based on clindamycin and even with trimethoprim sulfametazole in communities with high prevalence of Methicillin Resistant Staphylococcus for handling soft tissues minor infections (Jorgenser, 2000; Agnoletti *et al*., 2014). In the present study it was found 7% of MRSA strains resistant to clindamycin, indicating the risk of acquiring these strains through food.

CONCLUSIONS

7.5% of *Staphylococcus aureus* reported were MRSA and 33.8% were MSSA, in a total of 25% of the sampled establishments. The type of analyzed meat that obtained the highest percentage of MRSA was ground beef with 66%. MRSA strains are distribute in the three locations in the city of Cartagena, 40% in the localidad Virgen y Turística, 40% in the localidad Industrial de la Bahía and 20% in the localidad Histórica y del Caribe. It was found a marked antibiotic resistance in the methicillin resistant *Staphylococcus aureus* strains and in strains that carried the PVL toxin, obtaining a antibiotic multidrug resistance. It was confirmed circulation and possible spread of MRSA strains capable of producing genes encoding PVL toxin in meat products, which represents a risk to meat consumers in the city of Cartagena.

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