International Journal of Food Microbiology 209 (2015) 34–38

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Foods from black market at EU border as a neglected route of potential methicillin-resistant *Staphylococcus aureus* transmission

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ARTICLE INFO

Keywords:
Methicillin-resistant *Staphylococcus aureus*
LA-MRSA
Food
Black market
Border

ABSTRACT

The illegal entrance of foods to EU through black markets at the EU borders can constitute a neglected route of dissemination of foodborne pathogens, and in particular of methicillin-resistant *Staphylococcus aureus* (MRSA). In this study, we have assessed the presence of MRSA in foods sold in a black market at an EU border (the south-east part of Romania, on the border with Republic of Moldavia). We performed a search for MRSA among 200 food samples collected from 2012 to 2013. All *S. aureus* were studied by pulsed-field gel electrophoresis (PFGE) and antimicrobial susceptibility testing. MRSA isolates were further characterized by multilocus sequence typing (MLST) and SCC*mec* typing, and tested for the presence of Panton–Valentine leukocidin (PVL) virulence factors. Overall, 32 *S. aureus* isolates were recovered from 16 food samples (8%). One isolate detected in a pork lard sample was MRSA (0.5%). PFGE with the restriction enzyme *Sma*I revealed 12 genotypes among the 32 *S. aureus* isolates. The MRSA isolate belonged to sequence type 398, harbored SCC*mec* type V, tested negative for the presence of the PVL genes and was resistant to ciprofloxacin, tetracycline and cefazolin, besides all β-lactams. Among 31 methicillin-sensitive *S. aureus* (MSSA), 29% were resistant to penicillin, 9.7% to tetracycline and 3.2% to ciprofloxacin. In conclusion, in this study we report the presence of livestock-associated MRSA in foods sold in a black market at an EU border: ST398-MRSA-V. These results confirm the potential role of food in the dissemination of MRSA lineages among population, and the potential role of illegally introduced food to EU in the prevalence and evolution of MRSA clones in the community.

1. Introduction

It is estimated that *Staphylococcus aureus* produced an average of 241,148 episodes of domestically acquired foodborne illnesses caused in United States (Scallan et al., 2011), representing an important cause of food poisoning. In addition, this foodborne pathogen cause nosocomial invasive infections ranging from mild skin and soft tissue infections to life-threatening diseases such as septicaemia, endocarditis and necrotizing pneumonia (Lowy, 1998). It has been estimated that around 30% of healthy human individuals are colonized by this opportunistic pathogen (Graveland et al., 2011). *S. aureus* frequently harbor antibiotic resistance determinants which complicate treatment and significantly increase the associated costs. Currently, methicillin-resistant *S. aureus* (MRSA) is distributed worldwide and constitutes a major concern in human health because of its complex epidemiology and its ability to acquire novel antibiotic resistance mechanisms.

MRSA was first described in 1960, within a year after the inclusion of methicillin in the clinical practice to treat infections caused by the emergence of penicillin-resistant *S. aureus* (Jevons, 1961). Its presence was restricted to the clinical environment initially, but at the end of the past decade first cases of MRSA infections in the community were reported affecting people who exhibited no typical risk factors of hospital acquisition (Otter and French, 2010). Until the beginning of this century, MRSA had been rarely reported in livestock. It was first described in 1975, and after that, only sporadic cases were reported in the following 25 years. From 2005 onwards, MRSA belonging to sequence type (ST)

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; SCC*mec*, staphylococcal cassette chromosome *mec*; PLV, Panton–Valentine leukocidin; CA-MRSA, community-associated MRSA; LA-MRSA, livestock-associated MRSA; PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing; ST, sequence type

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http://dx.doi.org/10.1016/j.ijfoodmicro.2014.11.015
0168-1605/

398 was observed to colonize pigs and people professionally exposed to pig farming in several European countries (Voss et al., 2005; Witte et al., 2007). Later studies revealed the presence of that lineage in other food-producing animals, and therefore was designated livestock-associated MRSA (LA-MRSA).

Emergence of MRSA in food-producing animals has provoked a great concern in the presence of MRSA in associated foodstuff due to the potential for dissemination in the population. The role of food as vehicle of human MRSA dissemination is deemed to be low by the European Food Safety Authority (EFSA). However, foodborne MRSA infections has been formally demonstrated in several occasions (Jones et al., 2002; Kluytmans et al., 1995), and food can be a successful route for transmission of MRSA lineages. In this study, we have evaluated the presence of MRSA in food illegally introduced and sold in a black market at an EU border (the southeast part of Romania, on the border with Republic of Moldavia). This information provides an overview on the potential risk via foods introduced from non-EU countries as personal goods, which are then illegally sold to EU consumers, consequently defining a neglected route of transmission, as well as to reveal the role that it could play in the prevalence and dissemination of MRSA.

2. Material and methods

2.1. Sampling and bacterial isolates

A total of 200 samples were taken from July 2012 to February 2013 in a black market in Galati, Romania, a place close to the border with Republic of Moldavia. The food samples were transported under refrigeration to the laboratory and their microbiological analysis started in the same day they were collected. The food samples were classified into four categories: milk and dairy products (36%), fish and fish products (31%), meat and meat products (20%), and 13% others (e.g. eggs, biscuits, spices). Sampling was done randomly and the food samples were tested for the presence of *S. aureus* in accordance with ISO 6888:2 (ISO, 1999) and confirmed by real-time PCR (Trnčíková et al., 2009).

2.2. Screening for the presence of MRSA

We assessed the presence of *mec*A and *mec*C in all *S. aureus* isolates by multiplex PCR as previously described (Stegger et al., 2012). *mec*C and *mec*A positive controls, as well as non-template controls were included in each run.

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the microdilution method following the recommendations and MIC breakpoints of the CLSI guidelines (2012). Susceptibility to the following 20 antimicrobial agents was tested: penicillin, oxacillin, amoxicillin/clavulanate, daptomycin, cefazolin, erythromycin, clindamycin, teicoplanin, vancomycin, ciprofloxacin, levofloxacin, amikacin, gentamicin, tobramycin, mupirocin, rifampin, tetracycline, fusidic acid, fosfomycin, linezolid and cotrimoxazole.

2.4. Genetic fingerprinting

Genetic characterization of all isolated *S. aureus* was carried out by pulsed-field gel electrophoresis (PFGE) as previously described (McDougal et al., 2003). PFGE patterns were analyzed with Bionumerics v.6.6 (Applied-Maths NV, Sint-Martens-Latem, Belgium) to describe genetic relationships among isolates. Dendograms were constructed using the Dice similarity coefficient and the unweighted pair group mathematical average (UPGMA) clustering algorithm with 1% in the tolerance and optimization values. The Simpson's index of diversity was calculated to assess the discriminative power of PFGE by using the Comparing Partitions website hosted at http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Home.

Multilocus sequence typing (MLST) of MRSA was performed as described elsewhere (Enright et al., 2000). Allelic profiles were assigned in the *S. aureus* MLST database hosted at http://saureus.mlst.net/. Information of the MRSA was submitted to that database.

2.5. Typing and subtyping of the SCC*mec* element

We determined the genetic structure of the SCC*mec* element by multiplex-PCR as previously described (Kondo et al., 2007). This molecular method allows the discrimination of the SCC*mec* types I, II, III, IV, V and VI, as well as the variants IA & IIIA.

2.6. Detection of Panton–Valentine leukocidin virulence factors

We investigated the presence of the PVL genes (*luk*S-PV & *luk*F-PV) by conventional PCR as described by Lina et al. (1999). Reference strain ATCC 49775 was used as positive control.

3. Results and discussion

The emergence of MRSA in food-producing animals has elicited a great concern in the last decade on the potential role of foods in the dissemination of MRSA lineages. Consequently, many studies have assessed the presence of this pathogen in food samples from different countries and animal origins. Prevalence of MRSA in foodstuff greatly varies depending on the animal and the country of origin. Thus, while pork showed the highest contamination rate in the USA and Canada, poultry did in the Netherlands and Denmark (Bhargava et al., 2011; Kluytmans, 2010). In this study we analyzed 200 food samples from a black market in Galati, Romania, a place close to the border with Republic of Moldavia, assessing the presence of MRSA in foods illegally introduced and sold in a black market closed to an EU border. Crossing the Romanian border with food is allowed just for low amounts as personal goods on distances that do not overpass 50 km, but very often these foods are illegally sold in black markets. There are 9 border crossings between Romania and Republic of Moldova and 5 Romanian cities situated at distances that are in accordance with the Romanian law 10/2010. In each of those cities, there are one or two markets where Moldavians are coming daily to sell foods. Food products sold in such places are not coming only from Moldavia, but also from Ukraine, Bulgaria and Russia. Those market are organized to sell fresh fruits and vegetables and do not have facilities for refrigeration. Despite the fact that local authorities installed adverts saying that food of animal origin is forbidden to be sold and make periodical controls of the activity taking place in these markets, eggs, fish and fish products, milk and dairy products, meat and meat products are sold daily. It is difficult to estimate the amounts of food from non-EU countries entering in that way, but the frequency of this phenomenon is constant and high. Furthermore, Reg (CE) 206/2009 regarding the introduction in the EC of personal foodstuff of animal origin bans the introduction of meat, milk and derived foodstuff if they are not from EU-countries or Croatia, Faeroe Islands, Greenland or Iceland.

Overall, 32 *S. aureus* isolates were recovered from 16 confiscated food samples (8%): 8 milk and dairy products, 5 fish products and 3 meat samples. Among them, one isolate (0.5%) recovered from pork lard sample was MRSA as harbored the *mec*A resistance determinant. None isolate harbored the *mec*C homologue. Genetic characterization of all 32 *S. aureus* by *Sma*I-PFGE provided a fingerprint pattern consisting on 13–17 DNA fragments of 20–670 kbp, approximately. Twelve genotypes were observed resulting in a Simpson's Index of Diversity of 0.909 (CI 95% 0.854–0.963), but no relationship among the pulsotype and the sample type or the date of confiscation was observed.