Molecular detection of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE) isolates in raw chicken meat

*Aklilu, E., Nurhardy, A. D., Mokhtar, A., Zahirul, I. K. and Siti Rokiah, A.

Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, 16100 Pengkalan Chepa, Kota Bharu Kelantan

Abstract

Multi-drug resistant staphylococci including methicillin-resistant *Staphylococcus aureus* (MRSA) and Methicillin-resistant *Staphylococcus epidermidis* (MRSE) are among the emerging pathogens and have become a threat to both human and animals. Foods of animal origin can easily be contaminated by these bacteria if handled unhygienically or exposed to contaminated environmental surfaces. The objective of this study was to investigate the occurrence of MRSA and MRSE in raw chicken meat sold at wet markets in Kota Bharu, Kelantan, Malaysia. One hundred fresh raw chicken meat samples were collected from three different wet markets in Kota Bharu, Kelantan. Routine isolation and identification, selective media (Brilliance MRSA2 agar), antimicrobial sensitivity test (AST), minimum inhibitory concentration test (MIC), and polymerase chain reaction (PCR) amplification of *nuc*A gene and the resistant gene, *mec*A were conducted. Based on bacteriology results and growth on selective media, MRSA and MRSE were detected in 43% (43/100) of the raw chicken meat samples. Using the PCR assay, 77% (34/43) isolates were positive for *nuc*A gene. The detection of these emerging multi-drug resistant bacteria in chicken meat intended for human consumption implies the potential contamination of food items by the bacteria which in turn may pose risk to the public health.

Introduction

Originally, MRSA was a nosocomial pathogen, but in the 1990s, it spread into communities worldwide (Persoons *et al*., 2009). Methicillin-resistance was also reported in *S. epidermidis*. Methicillin resistance is mainly attributed to the presence of *mec*A gene located on one of staphylococcal cassette chromosomes mec (SCCmec) (Hiramatsu *et al*., 2001). This gene encodes the production of the penicillin-binding protein PBP2a, which has a low binding affinity to β-lactam antibiotics. However, because of the heteroresistance of *S. aureus* (Mackenzie *et al.*, 1993) it is often difficult to detect the presence of the resistance gene, *mec*A. Several reports have indicated the spread of MRSA in hospitals around the world and its global prevalence ranges from 23.3% to 73% (Diekema *et al.*, 2001). In recent years, MRSE has emerged as a major nosocomial pathogen (Miragaia *et al*., 2002). *Staphylococcus epidermidis* is a bacterium that constitutes a major component of the normal skin and mucosal microfloras of humans and is leading cause of device-associated infections in critically ill patients (Kozitskaya *et al.*, 2005). Among catheter-related and other foreign body infections 50 to 70% of the cases are caused by *S. epidermidis*. Biofilm formation by *S. epidermidis* enables the bacteria to adhere to foreign bodies like catheter and environmental surfaces. This biofilm formation is mediated by a range of outer surface proteins, including staphylococcal surface proteins (SSP1 and -2), autolysin proteins (AtlE), and an accumulation-associated protein (Aap) (von Eiff *et al.,* 2002).

Few studies have been reported the detection of MRSA in food including raw chicken meat (Lee, 2006). Limited number of reports on MRSA in domestic animals and chicken meat has been reported (Aklilu *et al*., 2010; Saleha and Zunita, 2010). Although there are reports confirming the detection of MRSE in human and animals elsewhere in the world including Malaysia (Baptiste *et al.*, 2005; Iorio *et al.*, 2011; Zaidah *et al*., 2011; Nurul Azirah *et al*., 2014). However, there are no reports as to the presence, detection and molecular characteristics of these bacteria in chicken meat. Therefore, the objectives of this study were to detect and molecularly characterize MRSA and MRSE in raw chicken meat sold at wet markets in Kota Bharu, Kelantan, Malaysia.

*Corresponding author.
Email: *erkihun@umk.edu.my*
Tel: +60-97 771 7175

Materials and Methods

Isolation and identification of bacteria

One hundred raw chicken meat samples were collected from three (Taman Bendehara, Pasir Puteh, and Kota Bharu) wet markets. A piece of fresh raw chicken meat was transferred into a sterile sampling bag with normal saline (0.9% NaCl) and was transported to the laboratory in an ice box. The samples were soaked in 15 ml of normal saline (0.9%) at room temperature for 5 minutes and were shaken gently. Two millilitres of the rinsed samples were pipetted and put and added into 15 ml of Tryptone Soya Broth (TSB). The broth was incubated for 24 hours at 37°C to enrich the growth of *S. epidermidis*. The broth cultures were then cultured onto Columbia Horse Blood Agar and Mannitol Salt Agar (MSA). All inoculated plates were incubated aerobically at 37°C for 18-24 h. *Staphylococcus epidermidis* colonies were primarily identified based on colonial morphology and Gram-staining. The presumptive *S. aureus* colonies (yellow colonies) were then streaked onto MSA to obtain pure colonies of *S. aureus*. The pure colonies were then streaked again onto Brilliance MRSA 2 agar to further confirm the MRSA colonies.

Antibiotic sensitivity test (disc-diffusion method)

Antibiotic sensitivity tests were carried out for MRSA and MRSE isolates according to Clinical and Laboratory Standards Institute (CLSI, 2006). The antimicrobials tested were; Amoxycillin-clavulanic acid (AMC30), Ampicillin (AMP10), Gentamicin (CN10), Erythromycin (E15), Cefoxitin (FOX30), Linezolid (LZD30), Oxacillin (OX1), Ampicillin-sulbactam (SAM30), Teicoplanin (TEC30) and Vancomycin (VA30), Nitrofurantoin (FD10), Enrofloxacin (ENR5), Amoxicillin (AML10), Streptomycin (S10) and Mupiprocin (MUP20). Inocula were prepared from overnight MRSA and VRE cultures on blood agar. Pure colonies were emulsified into normal saline (0.9% NaCl) and turbidity of the suspension was equilibrated to 0.5 McFarland and spread onto Mueller Hinton Agar (MHA).

Minimum inhibitory concentration

Etest MICs of Vancomycin, Teicoplanin and Oxacillin for all isolates grown on Brilliance MRSA 2 agar were conducted. The MIC Etest strips were placed on MHA plates inoculated with 0.5 ml overnight cultures. The strip was pressed down with sterile forceps to insure complete contact with agar. After 15 minutes, the plates were aerobically incubated at 37°C for 24 h. The results were interpreted using criteria set by the CLSI (2006).

Preparation of genomic DNA and PCR procedures

The extraction of genomic DNA from the isolates was performed using DNeasy Blood and Tissue DNA purification kit (Qiagen) following the manufacturer's recommendations. Lysostaphin was added for effective extraction of DNA from the *Staphylococcus* spp. Extracted DNA was stored at -20°C until PCR was performed. PCR was performed using the following primers that would detect the *nucA* gene, specific to *S. aureus* and *mecA* gene, unambiguous to MRSA isolates. The *nucA* primers were: 5'-GCGATTGATGGTGATACGGTT-3' and 5'-AGCCAAGCCTTGACGAACTAAAGC-3', (279 bp) while the *mecA* primers were: 5'-AAAATCGATGGTAAAGGTTGGC-3' and 5'-AGTTCTGCAGTACCGGATTTGC-3' (533 bp). The PCR reactions were prepared in 50 µl volume, consisting 5µl PCR buffer, 2 µl MgCl$_2$, 1 µl dNTPs, 2 µl of each primer and 0.5 µl of Taq polymerase. The amplifications were conducted using MyCycler™ thermal Cycler (BioRad) programmed with the initial denaturation at 94°C for 10 min, 30 cycles (denaturation at 94°C for 30 s, annealing at 50°C for 45 s and extension at 72°C for 30 s) and final extension at 72°C for 10 min. The PCR products were separated by gel electrophoresis and the gels were analysed by using gel documentation system (GelDoc™, BioRad).

Results

Typical intense blue colonies of the isolate on Brilliance MRSA2 agar showed that the isolate were methicillin-resistant staphylococci. A total of 44 (44%) of methicillin-resistant staphylococci (MRS) were identified out of 100 fresh raw chicken meat sampled. Out of the 44 MRS isolates, 97.73% (43/44) and 2.27% (1/44) were MRSA and MRSE respectively. Out of the 44 isolates grown on Brilliance MRSA2 agar and identified as MRS 30% were resistant against oxacillin, 68% resistant against ampicillin, 45% resistant against linezolid and 30% resistant against erythromycin. However, 93% isolates were detected to be susceptible to gentamycin, 84% susceptible to ampicillin-sulbactam, 86% to amoxicillin, 82% susceptible to vancomycin, 73% susceptible to cefoxitin and 68% susceptible to teicoplanin. The MIC value for the isolate was below 4 µg/ml, which is below the 4 µg/ml breakpoint recommended for MRSA according to CLSI standards (CLSI, 2006).

Among all the isolates with positive growth

on Brilliance MRSA2 agar, 77.3% (34/44) of the isolates were positive for *nuc*A gene and thus were categorised as strains of *S. aureus*. However, 22.7% (10/44) of the isolates were negative for *nuc*A gene. Among the 44 culture positive isolates, only one isolate was positive for *mec*A gene. This isolate was negative for *nuc*A gene and was categorised as MRSE.

Discussions

Methicillin-resistant *Staphylococci*, particularly MRSA and MRSE are known to cause nosocomial infections. However, the recently increasing reports of these pathogens in animals and the community have indicated the spread of the bacteria outside the hospital environment. Because MRS strains are often resistant to a wide range of antimicrobials, the options for treating infections caused by these bacteria are getting more challenging. The current study revealed that 44% (44/100) of the chicken meat samples were tested positive based on growth on the selective media, Brilliance MRSA 2 agar. However, only one isolate was positive for the *mec*A gene and the same isolate was negative for the *nuc*A gene indicating that it was MRSE as evidenced by colonial morphology and growth on MSA. Previous studies conducted in Japan have reported low level of *mec*A positive *S. aureus* (2/444) isolates from retail raw chicken meat (Kitai *et al.*, 2005). Likewise, low level of MRSA was reported from a study conducted on raw chicken meat in South Korea (Lee, 2006). In the current study, all the 34 *S. aureus* grown on Brilliance MRSA 2 agar were negative for *mec*A gene. However, one of the isolate from 10 *S. epidermidis* isolates grown on the same media was positive for *mec*A gene. The discrepancy between the growth on selective media and PCR detection of *mec*A gene can be attributed to several factors. Absence of the *mec*A gene in this study can be attributed to several factors since there are other mechanisms that are non-mec dependent which contribute individually or in combination towards antibiotic resistance in staphylococci strains (Berger-Bachi, 1995; Berger-Bachi and Tschierske, 1998). According to Chambers (1997), the overproduction of normal PBP with an altered binding capacity or other unidentified factors potentially contribute to the rise of methicillin resistance in *mec*A negative *S. aureus* strains. Moreover, the Brilliance MRSA 2 selective agar used in this study does not exclude growth of *S. aureus* that are hyperproducers of penicillinase (Nahimana *et al.*, 2006).

Antimicrobial Susceptibility testing using disc diffusion showed that only 30% (13/44) and 27%

(12/44) of isolates were resistant to oxacillin and cefoxitin respectively. The MIC results were ≤ 4 μg/ ml for all the isolates. These findings indicate that the resistant level by using disc diffusion test and MIC in MRS strains might be related to the presence or absence of mecA gene. Food stuff such as chicken meat can be contaminated by different pathogenic bacteria including MRSA and MRSE. Such contaminations may occur due to environmental contamination by the bacteria or because of unhygienic handling of the meat during slaughtering, retail or food preparation. Staphylococci are normal flora on the human body surfaces and are among the most environmentally ubiquitous bacteria. Moreover, staphylococci are able to form biofilm on inert materials used in the food-processing industry as such foodstuffs can be one of the possible sources of multi-resistant *S. epidermidis* strains (Jaglic *et al.*, 2010). Although the risk of human infection with MRS from contaminated meat chicken is often considered minimal, its potential risk to the public health cannot be undermined. So far the detection of MRSA in food stuff such as chicken meat has been given much of the emphasis and there are no reports as to the isolation of MRSE from chicken meat to the best of our knowledge. The fact that MRSE strain was detected in chicken meat in this study signifies the importance of further investigation on MRSE's role in food contamination and on its public health implications in this regard. However, further studies supported by molecular typing to trace the source and spread of the bacteria are recommended.

Acknowledgements

Reference

Aklilu, E., Zunita, Z., Hassan, L. and Chen, H. C. 2010. Phenotypic and Molecular characterization of Methicillin-resistant *Staphylococcus aureus* isolated from cats and dogs at university veterinary hospital. Journal of Tropical Biomedecine 27: 483-492.

Baptiste, K. E., Williams, K., Williams, N. J., Wattret, A., Clegg, P. D., Dawson, S. J., Corkill, E., O'Neill, T. and Hart, C. A. 2005. Methicillin-resistant staphylococci in companion animals. Emerging Infectious Diseases 11: 1942-1944.

Berger-Bachi, B. 1995. Factors affecting methicillin resistance in *Staphylococcus aureus*. International Journal of Antimicrobial Agents 6: 13-21.

Berger-Bachi, B. and Tschierske, M. 1998. Role of Fem factors in methicillin resistance. Drug Research Updates 1: 325-335.

Chambers, H. 1997. Methicillin resistance in staphylococci: molecular and biochemical basis and clinic implications. Clinical Microbiology Reviews 10: 781-791.

CLSI. 2006. Performance Standards for Antimicrobial Disk Susceptibility Tests; Sixteenth Document M100-S16. International Supplement, Clinical and Laboratory Standards Institute, Wayne, PA, USA.

Diekema, D. J., Pfaller, M. A., Schmitz, F. J., Smayevsky, J., Bell, J., Jones, R. N. and Beach, M. 2001. SENTRY Participants Group: Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected i n the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997–1999. Clinical Infectious Disease 32: 114-132.

Hiramatsu, K., Cui, L., Kuroda, M. and Ito, T. 2001. The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. Trends in Microbiology 9: 486-493.

Iorio, N. L. P., Azevedo, M. B., Frazão,V. H., Barcellos, A. G., Barros, E. M., Pereira, E. M., de Mattos, C. S. and dos Santos, K. R. N. 2011. Methicillin-resistant *Staphylococcus epidermidis* carrying biofilm formation genes: detection of clinical isolates by multiplex PCR. International Microbiology 14: 13-17.

Jaglic, Z., Michu, E., Holasova, M., Vlkova, H., Babak, V., Kolar, M., Bardon, J. and Schlegelova, J. 2010. Epidemiology and characterization of *Staphylococcus epidermidis* isolates from humans, raw bovine milk and a dairy plant. Epidemiology a n d Infection. 138: 772-782.

Kitai, S., Shimizu, A., Kawano, J., Sato, E., Nakano, C. and Uji, T. 2005. Characterization of methicillin-resistant *Staphylococcus aureus* isolated from retail raw chicken meat in Japan. Journal of Veterinary Medicine 67: 107-110.

Kozitskaya, S., Olson, M. E., Fey, P. D., Witte, W., Olsen, K. and Ziebuhr, W. 2005. Clonal Analysis of *Staphylococcus epidermidis* Isolates Carrying or Lacking Biofilm-Mediating Genes by Multilocus Sequence Typing. Journal of Clinical Microbiology 43: 4751-4757.

Lee, J. H. 2006. Occurrence of methicillin-resistant Staphylococcus aureus strains from cattle and chicken, and analyses of their *mec*A, *mec*R1 and *mec*I genes. Veterinary Microbiology 114: 155-159.

Mackenzie, A. M., Richardson, H., Missett, P., Wood, D. E. and Groves, D. J. 1993. Accuracy of reporting of methicillin-resistant *Staphylococcus aureus* in a provincial quality control program: a 9-year study. Journal of Clinical Microbiology 31: 1275-1279.

Miragaia, M., Couto, I., Pereira, S. F. F., Kristinsson, K. G., Westh, H., Jarløv, J. O., Carriço, J., Almeida, J., Santos-Sanches, I. and de Lencastre, H. 2002. Molecular Characterization of Methicillin-Resistant *Staphylococcus epidermidis* Clones: E v i d e n c e of Geographic Dissemination. Journal of Clinical Microbiology 40: 430-   438.

Nahimana, I., Francioli, P. and Blanc, D. S. 2006. Evaluation of three chromogenic media (MRSA-ID, MRSA-Select and CHROMagar MRSA) and ORSAB for surveillance cultures of methicillin-resistant *Staphylococcus aureus*. Clinical Microbiology and Infection 12: 1168-1174.

Nurul Azirah, M. S., Hassriana, F. S., Hui-min, N. and Salasawati, H. 2014. First report on the molecular epidemiology of Malaysian *Staphylococcus epidermidis* isolated from a University Teaching Hospital. BMC Research Notes 7: 597.

Persoons, D., Van Hoorebeke, S., Hermans, K., Butaye, P., de Kruif, A., Haesebrouck, F. and Dewulf, J. 2009. Methicillin-resistant *Staphylococcus aureus* in poultry. Emerging Infectious Diseases 15: 452 -453.

Saleha, A. and Zunita, Z. 2010. Methicillin resistant *Staphylococcus aureus* (MRSA): An emerging veterinary and zoonotic pathogen of public health concern and some studies in Malaysia. Journal of Animal and Veterinary Advances 9: 1094-1098.

von Eiff, C., Peters, G. and Heilmann, C. 2002. Pathogenesis of infections due to coagulase-negative staphylococci. Lancet Infectious Diseases 2: 677-685.

Zaidah, A. R., Siti Hawa, H., Siti Asma, H., Sabariah, O. and Siti Suraiya, M. N. 2013. The significance of coagulase-negative staphylococci bacteraemia in a low resource setting. Journal of Infection in Developing Countries 7: 448-452.