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Frequency of methicillin resistant *Staphylococcus aureus* in the noses of Malaysian chicken farmers and their chicken

Neela, V.[1]; Ghaznavi-Rad, E.[2*]; Ghasemzadeh-Moghaddam, H.[1]; Nor Shamsudin, M.[1, 3]; van Belkum, A.[4] and Karunanidhi, A.[1]

[1]Department of Medical Microbiology, Faculty of Medicine and Health Sciences, University Putra Malaysia, Serdang 43400, Selangor, Malaysia; [2]Departments of Microbiology and Immunology, Faculty of Medicine, Arak University of Medical Sciences, Arak, Iran and Molecular and Medicine Research Center, Arak University of Medical Sciences, Arak, Iran; [3]Marine Science Laboratory, Institute of Bioscience, University Putra Malaysia, Serdang 43400, Selangor, Malaysia; [4]Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Gravendijkwal 8 230 3015 CE Rotterdam, The Netherlands and BioMérieux, La Balme les Grottes, France

[*]**Correspondence:** E. Ghaznavi-Rad, Departments of Microbiology and Immunology, Faculty of Medicine, Arak University of Medical Sciences, Arak, Iran. E-mail: e.ghaznavirad@arakmu.ac.ir

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Summary

The prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin sensitive *Staphylococcus aureus* (MSSA) carriage among poultry and poultry farmers in Malaysia is largely unknown. In the current investigation, chickens and chicken farmers from 30 chicken farms were screened for MRSA and *S. aureus* carriage. The genetic characteristics of the isolates were determined through multi locus sequence typing (MLST), *Staphylococcus* protein A (*spa*) typing and virulent gene profiling. The outcome of the study showed lack of MRSA and extremely low *S. aureus* prevalence (n=7 of 503, 1.4%) among chicken flocks and the poultry farmers in Malaysia. *Staphylococcus aureus* isolates belonged to 4 sequence types (ST): ST97 (*spa* type t359), ST1179 (t359), ST 692 (t2247) and ST188 (t189). It can be concluded that MRSA/MSSA prevalence is very low among chicken and chicken farmers, human and chicken cross transmission of *S. aureus* does not seem to be a threat in Malaysia.

Key words: Chicken, *Staphylococcus aureus*, MRSA, Malaysia

Introduction

During recent years there have been several reports on the isolation of methicillin resistant *Staphylococcus aureus* from poultry farms or slaughter houses, carcasses, or food of poultry origin. Studies have shown that transmission of *S. aureus* and MRSA can occur from human to animal and *vice versa* and direct exposure to MRSA-positive animals may lead to transmission to humans (Persoons *et al*., 2009; Lim *et al*., 2010).

Infections acquired in the community can also come from animals. Animals may be a reservoir for human infection by MRSA since many different species can carry or have infections due to MRSA. Meanwhile, molecular typing methods have been used to track the sources and transmissions of pathogenic bacteria, thereby helping the establishment of national and global epidemiological data of pathogens like *S. aureus* and MRSA (Harmsen *et al*., 2003). Little information is available on the occurrence of MRSA in chickens. Methicillin resistance was first observed in *S. aureus* isolates from chickens in Korea (Lim *et al*., 2010). MRSA was later detected in broiler chickens in Belgium (Persoons *et al*., 2009). Our previous study (Neela *et al*., 2009) on MRSA from pigs in Malaysia has identified ST9 to colonize Malaysian pigs and pig handlers rather than the more traditional ST398. A recent report from The Netherlands has documented the isolation of

ST9 from chicken (Mulders *et al*., 2010). Because of the increasingly frequent isolation of MRSA from poultry and the association of ST9 MRSA with chicken in The Netherlands and also the fact that chicken are the main meat source in Malaysia, the current study was aimed to investigate the prevalence and characterization of *S. aureus* and MRSA in commercial broilers and laying chickens in Malaysia.

Materials and Methods

Sampling location

Birds and farm workers from thirty randomly selected commercial (non-exporting) poultry farms (15 broilers and 15 laying) from four districts (Kuala Selangor, Hulu Selangor, Gombak and Kuala Langat) in the state of Selangor of Malaysia were sampled in June 2010 by a trained veterinarian. Farms were located in an isolated area away from major towns or human dwellings. The distance from farm to farm ranged between 3 to 50 km within the districts. The farm sizes ranged from 10,000 to 200,000 birds.

Sampling procedure

An informed consent was obtained from the farmers for the use of their birds and from the farm workers for participation in the study. The sampling from chicken went as follows: one person held the chicken firmly while another person inserted the swab (110C Mini Tip Amies, COPAN Italia, Brescia, Italy) slowly "screwing" it into the nasal cavity (3-5 mm deep) of the bird (i.e. under the waxy, softer flap).

Only birds over 25 days in age were sampled. A total of 503 samples (420 from birds and 83 from farm workers) were collected, of which, 360 were from the nostrils of the birds (174 from layers and 186 from broilers), 60 from under the wings (30 from layers and 30 from broilers) and 83 from the nose of farm workers (VandenBergh *et al*., 1999). Under wings samples were taken from the same birds that were sampled for nasal culture.

Three workers from each farm were sampled, except for seven broiler farms, from which only two participated. All farmers and birds included in the study were healthy and did not have any medical complication at the time of sampling.

Isolation of *S. aureus* from chicken and farm workers

All sampled swabs were transported to the microbiology laboratory on the same day of sampling in an icebox and processed immediately. In the laboratory the swabs were incubated at 37°C overnight in an enrichment media (Muller-hinton broth containing 6.5% NaCl) and then sub-cultured on BBL CHROM agar *S. aureus* (CSA, BD Diagnostics) using a three-streak dilution method. The results were read after 24 h of incubation at 35°C. Growth of colonies showing mauve coloration was considered to be indicative for *S. aureus.* Mauve colour colonies were confirmed as *S. aureus* by coagulase test, gram staining and the Sa442 PCR (Martineau *et al*., 1998). *Staphylococcus aureus* isolates were tested for methicillin resistance by latex agglutination test (Denka Seiken Co., Ltd., Tokyo, Japan), cefoxitin disk diffusion (CLSI, 2007) and *mecA* PCR (Oliveira and Lencastre, 2002). The antibiotic susceptibility pattern of the isolates was tested by using the Kirby-Bauer disk diffusion method and the results were interpreted according to the CLSI guidelines (CLSI, 2007).

Molecular typing of *S. aureus* and virulent gene characterization

For all strains, the *spa* types and MLST were determined. Briefly, Chromosomal DNA was extracted using the DNeasy Kit (Qiagen Inc.) according to the manufacturer's instructions. All MSSA isolates, irrespective of their sources, were subjected to spa typing according to the method of Shopsin *et al*. (1999) using the primers SpaF (AGACGATCCTTCGGTGA) and SpaR (CAGCAGTAGT-GCCGTTTG). The amplified *spa* gene fragment was purified and sequenced. Spa types were assigned by using StaphType software (version 1.5, Ridom GmbH, Würzburg, Germany), as described by Harmsen *et al*. (2003) (http://spaserver.ridom.de). MLST

was performed in three steps which include:
(i) Amplification of seven housekeeping genes
(ii) Purification and sequencing of PCR product
(iii) Analysis of the sequences through MLST database to obtain sequence types (ST's) according to the program available from the MLST Web site (http://www.mlst.net).

All isolates were screened for the presence of the virulence genes Panton-Valentine leukocidin (*pvl*), fibronectin-binding protein A (*fnb A*), collagen binding adhesion (*cna*), *S. aureus* enterotoxin *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *tsst*, *eta*, *etb*, toxic shock syndrome toxin (tsst), exfoliative toxin (*eta*, *etb*), intercellular adhesion operon A (*Ica A*), intercellular adhesion operon B (*Ica B*), accessory gene regulator (*agr*) and arginine catabolic mobile element ACME-*arcA* (Ghaznavi-Rad *et al*., 2010).

Results

Staphylococcus aureus isolation from chicken and farm workers

Among the 420 swabs (nasal and under wings) from chicken, *S. aureus* was isolated from the nose of 4 (0.95%) birds (all from layers). No *S. aureus* was isolated from the broilers and under wing swabs. On the other hand, of the 83 farm workers, three (3.61%) were positive for *S. aureus*. As the carriage rate was very low, the swabs were tested twice to reconfirm the results. Three of the 4 layer birds were from the same farm. Except for one worker, all other chicken or workers colonized with *S. aureus* were from the same district, Kuala Kubu, but from different farms. Colonized birds were found only in 2 of the 15 layer farms. None of the isolates proved to be MRSA.

Antibiotic susceptibility pattern

Susceptibility testing revealed 100% susceptibility towards clindamycin, erythromycin, fusidic acid, gentamicin, trimethoprim-sulfamethoxazole, mupirocin, linezolid, vancomycin, quinupristine-dalfopristine, tigecycline, teicoplanin, netilmicin, penicillin, cephalexin, bacitracin, chloramphenicol, rifampicin, and cloxacillin. All isolates from the birds were ciprofloxacin resistant.

Molecular clonal types

The finding of molecular typing has been shown in Fig. 1 and summarised in Table 1. Molecular typing data indicates that *S. aureus* isolates belonged to 4 sequence types: ST97 (*spa* type t359), ST1179 (t359), ST 692 (t2247) and ST188 (t189). Isolates from all layer birds irrespective of the farms had the same sequence type (ST692) and spa type (t2247). Similarly all workers from the layer farms were colonized with t359 *S. aureus*, but had different sequence types (ST 97 and ST 1179 [a single locus variant of ST 97]). One *S. aureus* was isolated from a worker in the broiler farm, and appeared to be ST 188 (t189).

Virulence genes profile

Virulence gene analysis revealed that all isolates harboured *icaA*, *icaD* and *fnb*, except that the t188 strain carried *cna* instead of *fnb*. Superantigens (SAgs) *sec* and *tsst* were detected only in ST 1179 (human isolate). None of the isolates carried ACME-

Table 1: Characteristics of *S. aureus* isolates from poultry and farm workers in Malaysia

No.	Location	Source	Farm	*spa*	*ST*	*seb*	*sec*	*seg*	*sei*	*Seh*	*tst*	*eta*	*etb*	*icaA*	*icaD*	*fnb*	*cna*	*agr*
1	Kuala Kubu	Layer	Layer 5	t2247	ST692									P	P	P		I
2	Kuala Kubu	Layer	Layer 5	t2247	ST692									P	P	P		I
3	Kuala Kubu	Layer	Layer 5	t2247	ST692									P	P	P		I
4	Kuala Kubu	Layer	Layer 4	t2247	ST692									P	P	P		I
5	Kuala Kubu	Worker	Layer 1	t359	ST97									P	P	P		I
6	Kuala Kubu	Worker	Broiler 8	t189	ST188									P	P		P	I
7	Kuala Selangor	Worker	Layer 3	t359	ST1179		P				P			P	P	P		I

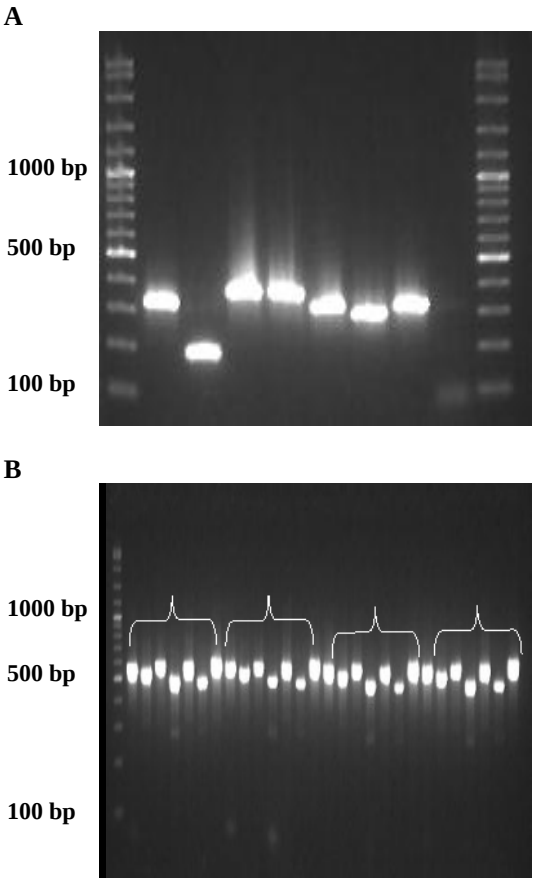

Fig. 1: (A) *spa* gene amplification from *S. aureus* isolated from poultry and farm workers. (B) Representative *S. aureus* strains from poultry and farm workers for amplification of seven MLST housekeeping genes

arcA, *pvl*, *eta*, *etb*, or enterotoxin genes (SEgs) *sea*, *seb*, *seg*, *sei*, and *seh*. All isolates where of *agr* type I.

Discussion

The fact that we did not detect any MRSA in poultry or poultry farmers is especially noteworthy and indicates that MRSA is absent or present only in low numbers among Malaysian flocks. In our earlier studies conducted on determining MRSA prevalence in the community and the hospital, MRSA was found to be generally low outside the hospital in Malaysia (Choi *et al*., 2006; Ghaznavi-Rad *et al*., 2010).

The extremely low prevalence of *S. aureus* in the current investigation prompted us to investigate whether there are any *S. aureus* inhibitors in the nose of chicken. A simple inhibition assay was conducted, where a streak of chicken nasal swab was made on Muller Hinton agar lawned with *S. aureus* isolated from chicken. Absence of clear zone around the streak indicated the lack of *S. aureus* inhibitors in the chicken nose.

Antimicrobials are actively used as growth promoters and prophylactics on poultry farms in Malaysia. Although the chicken feed used in the farm contains antibiotics like chloramphenicol, erythromycin, sulfonamide, penicillin, lincomycin, spectinomycin, oxytetracycline, endofloxacitin and tylosin, these antibiotics are used as per the guidelines. A recent study on the sulphonamides (SAs) determination in chicken meat products from Malaysia showed that, the concentration of SAs is lower than the maximum residual limit (MRL) established in Malaysia (Choi *et al*., 2006). The prescribed usage of antibiotics in the chicken feed explains the reason for the low prevalence of *S. aureus* and absence of MRSA in chicken nose. However, the 100% resistance to ciprofloxacin among *S. aureus* warns against the proper use of fluroquinolones in poultry. The low prevalence of *S. aureus* among the workers is surprising as the *S. aureus* carriage among the healthy individuals in Malaysia varies from 23 to 33% (Neela *et al*., 2008; VasanthaKumari *et al*., 2009). However, the reason for low prevalence could be due to the limited number of workers screened, only 2 to 4 subjects from each farm were screened, and these subjects may not be carrying *S. aureus* in their nose.

Although small numbers (0.91%) of *S. aureus* are isolated from chicken, it should not be taken lightly as association of *S. aureus* with disease such as septicaemia, skeletal infections, bacterial chondronecrosis have been identified as the predominant cause of lameness in commercial broiler chicken flocks (McCullagh *et al*., 1998). In the current study, the 4 *S. aureus* strains isolated from layers (three from the same farm and one from another which is 40 km away), shared indiscriminate strains as shown by MLST and spa typing. This shows that there might be cross-transmission of strains among the flocks inside and outside the farm. As the two farms from which the

S. aureus of ST 692-t2247 were isolated were from the same district, it is not clear whether the ST692 is the most dominant poultry associated *S. aureus* in Malaysia. ST692 has been recently reported in Korea from chicken meat but as an MRSA (Lim *et al*., 2010).

Isolates from workers ST1179, ST 97 and ST 188 are of human origin and have been previously shown to be isolated from clinical cases in Malaysia. Sam *et al*. (2008) reported ST1179 as a multi susceptible MRSA that is isolated from line associated sepsis in a tertiary care hospital. But in Japan, ST1179 is one of the most common MRSA strains causing bovine mastitis (Hata *et al*., 2010). In Malaysia, this is the first study to show its isolation from healthy individuals who are working closely with livestock. ST188 is slowly starting to predominate the Asian region, since it has been identified among clinical cases causing a broad spectrum of clinical diseases as PVL positive MSSA and also as PVL positive MRSA (Kim *et al*., 2007; Ghaznavi-Rad *et al*., 2010). Virulence gene analysis showed that none of the isolates are positive for any of the enterotoxins or other SAgs except for ST1179 isolated from one of the workers who carried *sec* and *tsst* genes. TSST is a well known virulence factor in *S. aureus* that causes toxic shock syndrome in humans (Dinges *et al*., 2000). Both the SEgs and TSST-1 belong to the pyrogenic toxin SAgs (PTSAg) family. In contrast to traditional antigens, SAgs bind to the outside of the major histocompatibility complex class II molecules and form a complex with the Vβ chain of a T-cell receptor (TCR), which leads to the stimulation of T-cell proliferation in a nonspecific manner resulting in host immune system (Dinges *et al*., 2000; Chang *et al*., 2005). Hence, carriage of *tsst* and *sec* genes positive *S. aureus* by the poultry farmer is a risk factor for both birds and human.

In the present study, we report extremely low methicillin susceptible and resistant *S. aureus* prevalence among Malaysian poultry and the people handling them. Despite the fact that transmission of *S. aureus* and MRSA does not seem to be of importance in Malaysia, more studies should be performed in the future, including more farms from different states and more individuals with different risk factors.

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