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DETECTION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) FROM ANIMAL AND HUMAN ORIGIN IN BANGLADESH BY POLYMERASE CHAIN REACTION

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is defined by the presence of the *mec*A gene, which is considered to have been transferred horizontally from unknown bacterial species to *S. aureus*. The *mec*A gene which encodes an additional β-lactam-resistant penicillin-binding protein (PBP), termed PBP-2a (PBP-2') with reduced binding affinity for β-lactam compounds. We investigated distribution of the *mec*A gene in a total of 94 clinical strains of *S. aureus* isolated from both man and animal admitted in Bangladeshi medical hospital as well as Veterinary clinic. The *mec*A gene was detected by PCR in 25% of human clinical isolates of *S. aureus*, whereas not a single *mec*A gene was detected in animal isolates of *S. aureus*.

Key words: Methicillin-resistant *Staphylococcus aureus* (MRSA), *mec*A gene, Polymerase Chain Reaction (PCR), Animal & Human

INTRODUCTION

Antibiotic resistance in *S. aureus* was almost unknown when penicillin was first introduced in 1943. By 1950, 40% of hospital *S. aureus* isolates were penicillin resistant and by 1960, this had risen to 80% (Chambers, 2001). Today, *S. aureus* has become resistant to many commonly used antibiotics. In the UK, only 2% of all *S. aureus* isolates are sensitive to penicillin with a similar picture in the rest of the world, due to a penicillinase (a form of β-lactamase). The β-lactamase-resistant penicillins (methicillin, oxacillin, cloxacillin and flucloxacillin) were developed to treat penicillin-resistant *S. aureus* and are still used as first-line treatment.

Methicillin was the first antibiotic in this class to be used (it was introduced in 1959), but only two years later, the first case of methicillin-resistant *S. aureus* (MRSA) was reported in England (Jevons, 1961). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a specific strain of the *Staphylococcus aureus* bacterium that has developed antibiotic resistance to all penicillins, including methicillin and other narrow-spectrum β-lactamase-resistant penicillin antibiotics (Foster, 1996). The resistant strain, MRSA which was first discovered in the UK in 1961, now is widespread, particularly in the hospital setting. MRSA may also be known as oxacillin-resistant *Staphylococcus aureus* (ORSA) and multiple-resistant *Staphylococcus aureus*. Despite this, MRSA generally remained an uncommon finding even in hospital settings until the 1990's when there was an explosion in MRSA prevalence in hospitals where it is now endemic (Johnson *et al*., 2004). Worldwide, an estimated 2 billion people carry some form of *S. aureus*; of these, up to 53 million (2.7% of carriers) are thought to carry MRSA. In the United States, 95 million carry *S. aureus* in their noses; of these 2.5 million (2.6% of carriers) carry MRSA (Graham *et al.*, 2006).

The genetic determinant of methicillin resistance in MRSA is the acquired gene *mec*A, which encodes the low-affinity penicillin-binding protein 2A (PBP2A), which can function as a surrogate trans-peptidase in the presence of high concentrations of β-lactam antibiotics that inactivate the four high-affinity PBPs native to *S. aureus* (de Jonge and Tomasz, 1993). It has been established that the production of an additional penicillin-binding protein PBP-2' (PBP-2a), with low-affinity for beta-lactam antibiotics, is mainly involved in the mechanism of methicillin resistance of *S. aureus* (Utsui and Yokota, 1985).

While the PBP-2', which is encoded by a chromosomal structural gene designated as *mec*A, is usually induced by beta-lactam antibiotics, it is known to be constitutively produced in some MRSA(Song *et al.*, 1987 and Ubukata *et al.*, 1990).

There are limited reports on the epidemiological aspects of nosocomial infections in the animal hospitals and laboratory settings (Hartmann *et al.*, 1997, Koterba *et al.*, 1986 and Tomlin *et al.*, 1999). Although, faucity of reports on nosocomial spread of an MRSA infection in a veterinary hospital could be found (Seguin *et al.*, 1999), there have been Veterinary reports of MRSA infections in dairy herds with mastitis (Devriese and Hommez, 1975 and Revill, 2003) and in companion animals (Koterba *et al.*, 1986; Tomlin *et al.*, 1999 and Cefai *et al.*, 1994) and of an isolated incident in a horses and horse personnel (Hartmann *et al*., 1997 and Weese *et al.*, 2005). There is a Press Headlines such as "Hospital Superbug" MRSA spreads to animals (Revill, 2003) and MRSA on the risk in U. K. Veterinary clinics (Veterinary Times, October 2004) have focused attention on the potential risk of MRSA to companion animal health. Transmission of MRSA between humans and horses has also been suspected in a Veterinary teaching hospital in the United States (Seguin *et al.*, 1999) and in Canada (Weese *et al.*, 2005). The environment contributes to MRSA transmission (Udo *et al.*, 1996) and transmission of MRSA to humans also caused by consuming contaminated food products of animals (Lee, 2003). The research on the detection as well as prevalence of MRSA in animal population & differences in prevalence of MRSA between animal and human origin has never been studied in Bangladesh previously. Therefore, this research will focus on the distribution and prevalence of MRSA in animal and human origin in Bangladesh.

MATERIALS AND METHODS

Isolation and Identification of *S. aureus*

The bacteria was isolated & identified by cultural characteristics, colony morphology, Gram's stain and biochemical tests.

Detection of MRSA by PCR

MRSA was detected by PCR, as described previously by Kobayashi *et al*. (1994).

Extraction of DNA of *S. aureus*

A total of 100 µl TNE buffer was taken in eppendorf tube in which bacterial sample was added and mixed by vortexing, centrifuged at 10000 rpm for 1 minute. The supernatant was removed by micropipette & added 10 µl achromopeptidase enzyme(disrupting solution) with the pellet, mixed well by pipetting and incubated the tube at 40-42^0 C in water bath for 10 minutes. Removed from water bath, added 50 µl of 0.5 M KOH in the tube and mixed by vortex, kept at room temperature for 5 minutes. Finally 50 µl of 1M-Tris-Hcl (P^H 6.76) was added and mixed by vortex, centrifuged at 10000 rpm for 1 minute and collected supernatant which contains DNA used for PCR.

Polymerase Chain Reaction (PCR) for detection of *mecA* gene from *S. aureus*

A total amount of 79 µl de-ionized distilled water (DDW) was taken to the eppendorf tube, added 10 µl X10 reaction/PCR buffer (Roche Diagnostics, Germany) & 8 µl of 2.5 mM dNTP (Takara, Japan) to the tube. Then added 2 µl *mec*A primer,1 µl supernatant DNA sample and 0.5µl Taq Polymerase (5 units/µl, Roche Diagnostics, Germany) to the tube. Gently mixed by vortex and then kept tubes in thermal cycler. A thermal cycler was used to amplification of DNA. The cycling program included 30 cycles of a denaturing step at 94^{0}C for one minute, an annealing step at 55^{0}C for one minutes, and an extension step at 72^{0}C for two minutes. After completion of cycling program reactions were held at 4^{0}C. Based on the nucleotide sequences of *mec*A gene (Song *et al.*, 1987 and Berger-Bächi *et al.*, 1989), the oligonucleotide primers were designed (Table 1) and target genes were synthesized by PCR.

Table 1. Sequences of oligonucleotide primers and their location in the *mecA* gene

Target gene	Primer name		Nucleotide sequence (5'-3')	Product length (base pairs)	Location (nucleotide numbers)
mecA	Mec-A1	(+)	AAAATCGATGGTAAAGGTTGGC	533	1282-1303
	Mec-A2	(-)	AGTTCTGCAGTACCGGATTTGC		1739-1814

Preparation of 1% Agarose gel for electrophoresis

Two grams agarose was dissolved into a 200 ml TAE buffer in a flask and was heated by microwave oven for 1-2 minutes up to transparent. A total of 20µl of 10 mg/ml ethidium bromide was added to 200 ml melted agarose and then melted agarose was poured on the gel casting tray and placed the comb of appropriate teeth and number and allowed to solidify on the bench (one hour at room temperature).

Amplified DNA sample preparation and electrophoresis

The comb was gently removed. The solidified gel was transferred to the electrophoresis chamber containing sufficient amount of TAE buffer with 30µl ethidium bromide keeping the gel horizontally. Loading dye (as required) placed on a piece of parafilm using adjustable micropipette (0.5 to 20µl) and 1µl of DNA size marker was mixed with drop of dye and loaded to the first hole of the gel. A total of 10µl of amplified DNA sample was added to next drop of dye and mixed well by pipetting subsequently loaded to the next hole and continued upto last hole of the gel following same procedure. The electrophoresis chamber was covered and the electrophoresis apparatus were connected to the power supply. Electrophoresis was carried out at 110 volt for 25 minutes to get the dye third fourth of the length. When DNA migrated sufficiently as judged from the migration of Bromophenol blue of loading buffer, the power supply was switched off and examined to detect the band under UV illumination (Figure 1).

Documentation of the DNA samples

After electrophoresis, the gel was taken out carefully from the gel chamber and the gel gently placed on the UV transilluminator in the dark chamber of the image documentation system. The UV light of the system was switched on; the image was viewed on the monitor, focused and saved in a dictate, as well as printed on thermal paper. The positive sample was detected by visualized band on the gel.

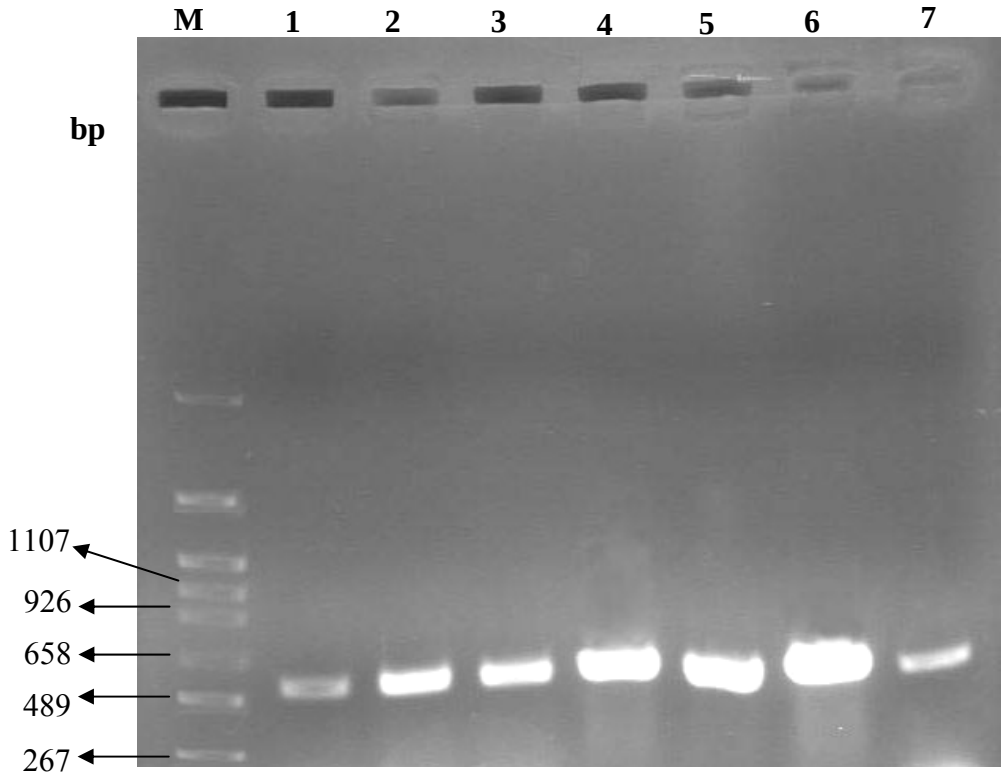

Figure 1. Agarose gel electrophoresis of PCR product amplified from *mec*A genes. These genes from seven Oxacillin resistant *S. aureus* strains. M = DNA marker fragments. Lane 1, 2, 3, 4, 5, 6 & 7 indicate the *mec*A positive samples. The DNA fragments of 533 bp were amplified from *mec*A gene.

RESULTS AND DISCUSSION

A total of 100 animal origin and 100 human origin specimens were included in this study. Among 100 animal origin specimens *Staphylococcus aureus* was 54 (54%). All the total animal origin *Staphylococcus aureus* were examined by PCR but no MRSA was detected.

The human origin samples were collected from hospitalized patients. Among 100 human origin specimens 40 were *Staphylococcus aureus* (Table 2). All the *S. aureus* of human origin specimens were examined by PCR. Out of 40 *S. aureus*, MRSA were 10 (25%). The detection rate of MRSA (n=10) isolated from different human origin specimens were given in Table 2. Majority (7.5%) of the MRSA were isolated from pus from skin infection followed by surgical wound swab, burn ulcer exudates and oral swab (5% each) and vaginal swab (2.5%).

Table 2. Detection of *S. aureus* and MRSA from different specimens of human origin

Type of specimen	Total no. specimen examined	Detection *of S. aureus*	MRSA (%)
Surgical wound swab	50	17	2 (5%)
Burn ulcer exudate	14	4	2 (5%)
Aural swab	11	8	2 (5%)
Pus from skin infection	19	9	3 (7.5%)
Diabetic ulcer exudate	5	1	-
Vaginal swab	1	1	1 (2.5%)
Total	100	40	10 (25%)

Methicillin-resistant *S. aureus* (MRSA) has become increasingly prevalent worldwide. Strengthening surveillance and screening of high-risk patients appears as an important component of effective infection control programme to limit the spread of MRSA in hospitals. In this context accurate detection of MRSA is essential. Antimicrobial susceptibility by disc diffusion or broth microdilution methods cannot detect MRSA those express low levels of oxacillin resistance. Recently many methods for detection of MRSA have been evaluated and are widely used in bacteriological laboratory. One of them has been designed as PCR detection of MRSA by detecting *mec*A gene. The present study attempted the detection of MRSA by PCR technique.

Two chromosal *mec* regulator genes *mec*RI and *mec*I have been identified (Hirasmatu *et al.*, 1992 and Tesch *et al.,* 1990). Surveys of the distribution of *mec* regulator genes among clinical isolates of methicillin-resistant staphylococci indicated that *mecI* encodes the repressor protein of the *mecA* gene and it is deleted or mutated in methicillin-resistant strains (Suzuki *et al.,* 1993). Although the mechanism of regulation of the *mec*A gene has not been completely elucidated, the presence of the *mec*A gene in staphylococci has been considered as a molecular basis for the identification of MRSA or methicillin-resistant coagulase negative staphylococci (CNS), even though the strain appears methicillin-sensitive by the measurement of minimum inhibitory concentration (MIC) (Hirasmatu *et al.*, 1992).On the basis of this findings, attempts have been made to identify MRSA by polymerase chain reaction (PCR) amplification of *mec*A gene fragments derived not only from isolated strains but also from clinical specimens directly (Higashiyama *et al.*,1993, Mukakami *et al.*, 1991 and Tokue *et al.*, 1991).

The *mecA* gene and the associated large (40 to 60-kb) *mec* element (Fontana, 1985; Hartman and Tomasz, (1986); Matsuhashi *et al.*, 1986; Matthews *et al.*, 1987; Reynolds and Brown, 1985 and Tesch *et al.*, 1988) are not native to *S. aureus* but were acquired from an extra species source by an unknown mechanism (Beck *et al.*,1986 and Pattee, 1990). The nature of the extra species source, i.e., the evolutionary origin of *mec*A and the formation of the *mec* element, has remained largely a matter of speculation (Archer and Niemeyer, 1994; EI Kharroubi *et al.*, 1991; Hirasmatu, 1995 and Piras *et al.*, 1993).

In the present study all the animal origin *S. aureus* (n=54) were examined by PCR for *mec*A gene but no *mecA* positive strain was detected. The probable reason behind this outcome that there is no oxacillin preparation in our market for animal therapy. But Kwon *et al.* (2006) isolated three pre-MRSA, one silent *mec*A-carrying methicillin susceptible *S. aureus* (smMSSA) from retail chicken meat, and three MRSA from hospitalized dogs in Korea although methicillin (oxacillin) was not used in animal husbandry or in animal hospitals in Korea that was not in agreement with present study. Therefore, further extensive study is required for elucidate this issue.

Methicillin-resistant *S. aureus* (MRSA) represents a major challenge to hospitals in all countries due to the emergence and spread of isolates with decreased susceptibilities to several antibiotic classes, in addition to methicillin and the other members of the β-lactam family. Molecular typing techniques applied to international collections of MRSA isolates have contributed to the understanding of the epidemiology and evolution of this infectious agent.

The prevalence of MRSA differs strongly throughout the countries. In the present study, no MRSA in animals was found while in human the isolation rate is 25%. In 1999-2000, 20% of the European blood isolates were MRSA which is agreement with present study. Another study from Bangladesh in human reported an isolation rate of MRSA as 12.5% (Hossain *et al.,* 2002) which is not in agreement with present study, which also indicates that the incidence of MRSA in our country is increasing day by day. The increasing incidence of MRSA observed in this study might be due to the fact that our specimens were taken from tertiary hospital where there is no authentic antibiotic policy to treat infectious patients. As a result, indiscriminate use of antibiotics is not less common. In addition, hospital environment are not adequately hygienic. Overcrowding of patients and attendants favors the spread of infectious agents. So hospitals acquired infection either in surgery or in medical wards are quite high. Due to infection, patients stay becomes prolong in hospital keeping under antibiotic therapy. All these factors mentioned above might be sufficient cause to increase the acquisition of resistance property among strains of *S. aureus.*

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