ISSN 1810-3030 (Print) 2408-8684 (Online)

Journal of Bangladesh Agricultural University

Journal home page: http://baures.bau.edu.bd/jbau

Molecular detection of methicillin-resistant *Staphylococcus aureus* (MRSA) in ornamental birds having public health significance

Sumaiya Binte Zaman[1], Md. Abdus Sobur[1], Md. Jannat Hossain[1], Amrita Pondit[1], Mst. Minara Khatun[1], Md Abu Choudhury[2], Md. Tawyabur[1], Md. Tanvir Rahman[1]✉

[1]Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh

[2]The University of Queensland, UQ Centre for Clinical Research UQCCR, Herston, Brisbane, Australia.

ARTICLE INFO

Article history:
Received: 11 January 2020
Accepted: 28 March 2020
Published: 30 June 2020

Keywords:
Ornamental birds,
Zoonoses,
Antibiotic resistance,
MRSA, VRSA, *mecA*

Correspondence:
Md. Tanvir Rahman
✉: tanvirahman@bau.edu.bd

ABSTRACT

There has been an increase in the demand for ornamental birds as pets in Bangladesh. However, issues of Antimicrobial Resistance (AMR) in ornamental birds remain poorly understood. Methicillin-resistant *Staphylococcus aureus* (MRSA) are major human health problem. Present study was designed to investigate the potentiality of ornamental birds as a source for MRSA that could transmit to human. A total of 70 samples were collected randomly from various ornamental birds and bird handlers working in the shops. A semi-structured questionnaire-based interview was also conducted with bird shop attendants to suspect the possible AMR origin and transmission. Isolation and identification of MRSA were based on culture, disk diffusion method followed by PCR. Hemolytic activities were tested on blood agar plates Among the 70 samples, 40 (57.14%) were found positive for *S. aureus.* Phenotypically 77.50% *S. aureus* were found resistant to oxacillin (methicillin), while by PCR, only ten (25%) isolates were found positive for *mecA* gene. Both the ornamental birds and bird handlers carried MRSA. Among the MRSA isolates, phenotypically six isolates were found resistant to vancomycin *e.g.,*VRSA (vancomycin-resistant *Staphylococcus aureus*).As far we know, this is the first report in Bangladesh describing molecular detection of MRSA from ornamental birds and bird handlers. From this study it is evident that ornamental bird carries MRSA that could transmit to human.

Introduction

The extensive use of antimicrobial agents in veterinary and human medicine, particularly often at sub-therapeutic doses for growth promotion and routine prophylactics in animal, has triggered to multidrug-resistant development among bacteria (Bitrus *et al.,* 2016). MRSA had been receiving public health attention for over a decade. The *mecA* gene is the key gene responsible for the resistance against methicillin. Any *S. aureus* carrying *mecA* therefore considered as MRSA.MRSA had acquired the *mecA* gene which encodes an alternative penicillin-binding protein 2α with reduced affinity for methicillin. This gene complex also allows cross-resistance to non-beta lactam antibiotics such as clindamycin, ciprofloxacin, cotrimoxazole, erythromycin and gentamicin (Nworie *et al.,* 2013). The genomes of some MRSA strains evolved further by enabling acquisition of *vanA* gene from *Enterococcus* which drives the emergence of vancomycin-resistant *Staphylococcus aureus* (VRSA), thus allowing a higher threat to antimicrobial chemotherapy and diagnostic microbiology (Okolie, 2013).

MRSA was reported as a potential zoonotic pathogen and has been isolated from a number of animal species such as dogs, cats, horses, sheep, pigs and birds worldwide (Becker *et al.,* 2002). These have also been detected in livestock and humans in Bangladesh (Haque *et al.,* 2011). Treatment for MRSA is very limited because of its resistance pattern and in most cases vancomycin is the most preferred antibiotics for its treatment (Pletz et al., 2010). However, decreased efficacy of vancomycin against *S. aureus* was first reported in 1997 from Japan time leading to the increased VRSA and vancomycin-intermediate *S. aureus* (VISA) (Control, 2000).

Antimicrobial resistance (AMR) in bacterial pathogens threatens global health through the spread of AMR bacteria and AMR genes among poultry, animals and humans. MRSA are priority two category pathogen as per WHO. Ornamental birds could be a potential source for MRSA for bird handlers and other people coming in contact with these birds (Briscoe *et al.,* 2008). Not much work has been carried out on the occurrence of MRSA

Cite this article

Zaman, S.B., Sobur, M.A., Hossain, M.J., Pondit, A., Khatun, M.M., Choudhury, M.A., Tawyabur, M., Rahman, M.T. 2020. Molecular detection of methicillin-resistant *Staphylococcus aureus* (MRSA) in ornamental birds having public health significance. *Journal of Bangladesh Agricultural University*, 18(2): 415–420. https://doi.org/10.5455/JBAU.80165

in ornamental birds. Here, we investigated the potentiality of ornamental birds as a source for MRSA that could transmit to human.

Materials and Methods

Collection of samples and data

The study was conducted during April to July, 2018 in Mymensingh city, Bangladesh. All the samples were collected on random basis from ornamental bird shops located in Mymensingh city, and bird handlers working there. A total of 64 cloacal and tracheal swab samples of different ornamental birds and 6 samples from hand washing of bird handlers (shop keepers) were collected aseptically and transferred immediately to the Bacteriology laboratory, Department of Microbiology and Hygiene, Bangladesh Agricultural University for bacteriological analysis. The summary of samples collected with their number and source are presented in Table 1. During the sample collection, a semi-structured questionnaire-based interview were also conducted with bird shop owners, and bird handlers working in the shop (n=30) focusing their KAP (knowledge, attitude and practices) on disease occurrence, antibiotics used, hygiene of the shop, types of birds available etc. to have some idea regarding AMR origin and transmission among birds and bird handlers.

Table 1. Number of samples collected from various ornamental birds and bird handlers with their origin

Name of the birds (Scientific name)	Sources	Number of samples	
		Cloacal swab	Tracheal swab
Dove (*Spilopelia chinensis)*	Bridge Moor	4	2
Parakeet (*Psittacula finschii)*	Chorpara	4	2
Parrot *(Loriculus vernalis)*	Chorpara	4	2
Shalik *(Acridotheres tristis)*	Bridge Moor	4	2
Diamond dove (Geopelia cuneate)	Kewatkhali	4	2
Munia *(Lonchurapunctulata)*	Kewatkhali	4	2
Java *(Padda oryzivora)*	Kewatkhali	4	2
Finch *(Fringilla coelebs)*	Bridge Moor	4	2
Mayna *(Gracula religiosa)*	Kewatkhali	4	2
Budgerigar *(Melopsittac usundulates)*	Chorpara	4	0
HiramonTiya *(Psittacula roseate)*	Bridge Moor	4	2
Sub total		44	20
Hand washing	Kewatkhali		6
Total			70

Isolation and identification of Staphylococcus aureus

Initially *Staphylococcus* spp. was isolated by culturing on Mannitol Salt agar (MSA). Yellow colored colonies on MSA were Gram's stained and the Gram-positive cocci bacteria having clustered arrangement was considered as *S. aureus* (Habibullah *et al.,* 2017). The genomic DNA from *S. aureus* was extracted by boiling method (Begum *et al.,* 2016). Final confirmation of detection of *S. aureus* was done by PCR targeting *nuc* gene as previously described (Bakeet and Darwish, 2014). All the PCR were done in a final 25 µl reaction with 12.5 µl master mixture 2X (Promega, USA), 2 µl genomic DNA (around 30 ng), 1 µl (100 pmol) each primer and 8.5 µl nuclease free water. Amplified products were analyzed by electrophoresis in 1.5% agarose gel. Ethidium bromide was used for staining the product and visualized under ultraviolet trans-illuminator (Biometra, Germany). A 100bp DNA ladder (Promega, USA) was used as molecular weight marker. The hemolytic activity of the isolated bacteria was detected following growth on blood agar as described by other (Habibullah *et al.,* 2017).

Antibiotic sensitivity test

Antibiotic sensitivity test was performed by disk diffusion test on Mueller-Hinton agar (Hodeida, India) plates having a concentration of bacteria equivalent to 0.5 McFarland standards (Bauer *et al.* 1966). The plates were incubated at 37°C aerobically for 18-24 hours to observe the results. Results of the antibiotic sensitivity tests were recorded as sensitive, intermediately sensitive, or resistant, and the zone of growth inhibition was compared with the zone size interpretative tables provided by the Clinical and Laboratory Standards Institute (CLSI, 2011). The antibiotics disc used to determine the antibiogram were methicillin (oxacillin) at the dose of 5µg/disc. In addition, gentamicin (10 µg), ciprofloxacin (10 µg), chloramphenicol (10 µg), oxytetracycline (30 µg) and vancomycin (30 µg) were also used in the antibiogram study. Any isolate showing resistance against oxacillin was considered as MRSA and resistance against vancomycin was considered as VRSA.

Molecular detection of methicillin-resistant S. aureus (MRSA)

Molecular detection of methicillin-resistant *S. aureus* (MRSA) was done by PCR using the standard primers and protocol (Habibullah *et al.,* 2017) targeting *mecA* gene. All the PCR were done in final 25 µl volume as described earlier.

Statistical analysis

All the data generated were incorporated into the excel sheet (MS-2013) and performed descriptive statistics using SPSS software (SPSS-22.0, IBM, USA) to compute the frequencies of MRSA.

Results

The study was carried out to determine the phenotypic and genotypic occurrence of MRSA in various ornamental birds and bird handlers in Mymensingh area, Bangladesh. As showed in Table 2, among the 70 samples 40 (57.14%) were found positive for *S. aureus* with highest occurrence in cloacal swab (60%). Phenotypically among the 40 *S. aureus,* 77.50% isolates were MRSA and 15.00% were VRSA respectively (Table 3). Isolation of MRSA was confirmed by PCR (Fig 1 and 2). The ornamental birds that were found positive for MRSA were Dove (*Spilopelia chinensis),* Parakeet (*Psittacula finschii),* Parrot *(Loriculus vernalis),* and Budgerigar *(Melopsittac usundulates).* MRSA and VRSA were detected not only in birds but also in hand washing. The highest resistance was against oxytetracycline (80.00%). This was followed by resistance against chloramphenicol (15.00%), ciprofloxacin (10.00%) and gentamicin (2.5%). Among the 40 *S. aureus* only 10 were found positive for *mecA* gene (Table 2). When six human samples originated from bird handlers working in the shop were analyzed only one was identified as MRSA. This human isolated MRSA was also found resistant to vancomycin phenotypically. All the isolated *S. aureus* were screened for their hemolytic activities. Among the 40 isolates 21 (52.50%) showed hemolysis on blood agar. During the study a questioner was used to gather various information from shop attendants on possible occupational health hazards associated with AMR in ornamental birds and identifying factors responsible for AMR in those birds. Among the 30 bird shop attendants 18 (60%) had chosen this profession due to economic and recreational purposes whereas others only for economic issue.

Table 2. Occurrence of methicillin resistant *S. aureus* in ornamental birds

Sample (n)	Positive for *S. aureus* (*nuc*) (%)	Occurrence of MRSA (*mecA*) (%) among the *S. aureus*	Hemolysis on blood agar (%) among the *S. aureus*
Cloacal swab (44)	25 (56. 81)	7 (28.00)	15 (60.00)
Tracheal swab (20)	12 (60)	2 (16.66)	5 (41.66)
Hand washing (6)	3 (50)	1 (33.33)	1 (33.33)
Total (70)	40(57.14)	10 (25.00)	21 (52.50)

Table 3. Resistant pattern of *S. aureus* isolated from ornamental birds

Source (n)	Resistant pattern to indicated antibiotics (%)					
	OX	CIP	O	C	GEN	VA
Cloacal swab (25)	21 (84)	2 (8)	21 (84)	3 (12)	1 (4)	5 (20)
Tracheal swab (12)	9 (75)	2 (16.66)	10 (83.33)	2 (16.66)	0 (0)	0 (0)
Hand Wash (3)	1 (33.33)	0 (0)	1 (33.33)	1 (33.33)	0 (0)	1 (33.33)
Total (40)	31 (77.50)	4 (10.00	32 (80.00)	6 (15.00)	1 (2.50)	6 (15.00)

Here, n= Number of *S. aureus,* OX=Oxacillin, CIP=Ciprofloxacin, O= Oxytetracycline, C= Chloramphenicol, GEN= Gentamicin, VA=Vancomycin.

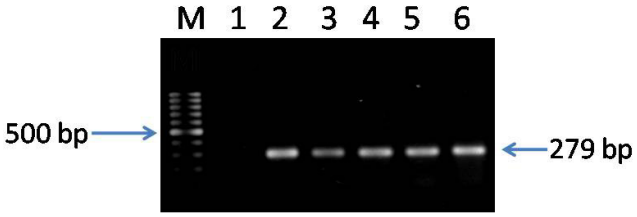

Fig. 1. Molecular detection of *nuc* gene of *S. aureus*. PCR amplification of *nuc* gene of *Staphylococcus aureus*. M=100 bp size DNA marker, Lane 1: Negatice control, Lane 2: Positive control, Lane 3 to 6: representative samples of *S. aureus* isolated from various ornamental birds

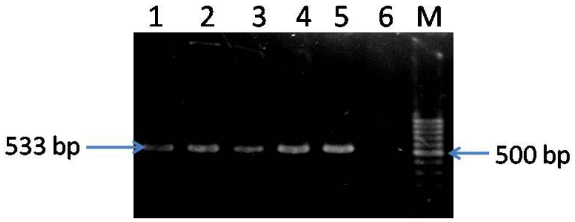

Fig. 2. Molecular detection of *mecA* gene of methicillin-resistant *S. aureus.* PCR amplification of *mecA* gene of methicillin-resistant *Staphylococcus aureus* (MRSA). Lane 1 to 4: representative samples of MRSA isolated from various ornamental birds, Lane 5: positive control, Lane 6: negative control, M=100 bp size DNA marker

Although the birds they sell are of multi-species and from different sources including importer, different markets, breeder farms and own breeding but none of them followed any isolation or quarantine. Around 83.3% of the shop also sell other poultry species. Most important observation was, 86.7% shop attendants have no knowledge about AMR, disease transmission (zoonoses). Most of them rear birds in both single and colony cage. Only three attendants washed hands before and after bird handling but none of the attendant used hand gloves or mask. Although 80% shop changed drinking water daily, none of them cleaned waterer, feeder and cages daily but weekly. Almost every shop faced disease symptoms including pox, sneezing, diarrhea and drowsiness in their birds occasionally (73.3%) and frequently (13.3%). Only 13.3% shops have been taken advices from veterinary doctor when birds became ill. All of the shop use antibiotics when birds were sick but only 10% of them completed antibiotic full course. Interestingly, 16.7% used antibiotic in feed and water for prophylaxis. Half of the shops used company feed and mixed grain followed by 33.3% (company) and 16.7% (mixed grain). Among the 30 attendants, 10 were experienced with coughing and sneezing (4), gastroenteritis (4) and abscess (2) and all 10 took antibiotics but only two completed antibiotic course.

Discussion

Globally MRSA have emerged as a significant public health problem both in human and veterinary medicine. MRSA are opportunistic pathogen. They are also zoonotic in nature and isolated from many animal species as well as humans (Persoons *et al.,*2009). It can cause a wide range of different infections in humans and poultry and some of which could be highly fatal as well (Zaheer *et al.,* 2017). VRSA among MRSA is an extra burden and it pays a great concern as vancomycin is the choice of drug against MRSA (Hasan *et al.,* 2016).

Involvement of MRSA in ornamental as an occupational hazard is not known. In this study we determined the occurrence of MRSA in ornamental birds for the first time in Bangladesh considering public health significance. MRSA are rare and less documented in ornamental bird (Hermans *et al.,*2000), instead most studies are focused on poultry. In Nigeria the occurrence of *S. aureus* in broilers and layers were detected as 49% and 51% respectively while MRSA as 6.1% and 15.3% respectively (Adeyeye and Adewale, 2013). Recently in Bangladesh three MRSA were detected out of 300 *S. aureus* isolated from chicken egg shell (Pondit *et al.,*2018). In this study we detected the occurrence of *S. aureus* and MRSA as 57.14% and 25% respectively. Some other authors recorded an incidence of 83.3% MRSA in the poultry attendants and 95% in chickens, much higher than our findings (Adeyeye and Adewale, 2013). These observed variations in the occurrence of MRSA could be due to diversity in avian species focused in these studies e.g., ornamental birds vs poultry, different management system of the poultry and ornamental birds including biosecurity, disease management system etc. It is also to note that not all methicillin resistant phenotype were found positive for *mecA* gene in PCR, suggesting that primers used here were not specific for these genes. Quality of extracted DNA could also be responsible for observing this phenomenon. Among the isolates 52.50% showed hemolysis on blood agar, indicating that other did not carry hemolysin producing genes.

Staphylococcal resistance to methicillin and other b-lactam antibiotics is due to the presence of the *mecA* gene or its *mecC* homologs, located in a mobile genetic element called the staphylococcal chromosomal cassette (*SCCmec)* (Katayama *et al.,* 2000). In this study PCR based molecular detection was performed successfully to identify MRSA from the ornamental birds, which is more sensitive than other methods used for the detection of MRSA.

Like many of the ornamental birds, in this study parrots were found positive for MRSA. Reports are available on the detection of MRSA from Psittacin birds in Belgium (Hermans *et al.,* 2000) and in parrot in Congo (Briscoe *et al.,* 2008). In parrot chronic history of feather plucking, self-mutilation or infection is thought to be due to MRSA infection. In man MRSA is frequently found in patients of staphylococcal food poisoning, post-operative wound infections, and pneumonia (Horan *et al.,* 1998). Hand washing of personnel handling ornamental birds in the shops was also found positive for MRSA in this study. These ornamental birds could be reservoir and source of MRSA for transmission to human, but the present study did not focus on the origin of these MRSA in human. According to some reports interspecies spread of MRSA is possible from birds to human and vice versa (Zaheer *et al.,* 2017).

It is interesting to note that among the 40 *S. aureus* six (15.00%) were found resistant to vancomycin phenotypically. VRSA was found in cloacal swab and hand washing. Occurrence of MRSA and VRSA in hand washing of bird handlers could be possible zoonosis. Moreover, all these six VRSA were also MRSA in nature, which is very alarming. VRSA infection in human is common especially in clinical samples. A study in Bangladesh found 11 VRSA among 21 MRSA isolated from burn wound infection (Hasan *et al.,*2016). Occurrence of VRSA in poultry is not uncommon. Report is available in Bangladesh on isolation of VRSA (17.39%) phenotype from *S. aureus* isolated from chicken egg shell. In Nigeria in poultry carcass the occurrence of VRSA was reported as high as 14.2% (Adeyeye and Adewale, 2013).

In Bangladesh, the antimicrobial agents most often used in poultry included β-lactam antibiotics, tetracyclines,

fluoroquinolones, macrolides, and trimethoprim/ sulfonamides. Resistance in the isolated MRSA were also noticed against oxytetracycline, ciprofloxacin, chloramphenicol, and gentamicin in this study. The highest resistance was observed against oxytetracycline (77.50%) and lowest against gentamicin (2.50%). In an earlier study in Bangladesh *Staphylococcus* spp. isolated from parrots in zoo were found highly sensitive to gentamicin (Akhter *et al.,* 2010). *S. aureus* isolated from chicken egg shell in Bangladesh were found resistance against gentamicin (34.78%) and oxytetracycline (39.23%) respectively higher and lower than present study.

There is no proper updated manual or guideline regarding management of ornamental birds in shop in Bangladesh. From our survey data we speculate that, the occupational health hazards of the bird handlers may be due to their ignorance on isolation, quarantine, personal hygiene and personal protection that are crucial to keep themselves safe from zoonoses like MRSA and VRSA. Large number of these shops keeps ornamental birds along with poultry, thus probably allowing the interspecies spread of MRSA and VRSA. Use of antibiotics in the feed supply to the birds and incomplete course of antibiotic treatment may be other contributing factors in observed AMR in the isolated *Staphylococcus* spp. that needs analysis of more data to be confirmed.

Conclusion

MRSA are important zoonotic pathogens. In this study MRSA were successful detected from ornamental birds for the first time in Bangladesh. Some of the isolates were found resistant to vancomycin (VRSA). Present findings demonstrate that ornamental birds are potential reservoir of MRSA which poses a threat to bird handlers. Further research work is now required to identify the possible origin of these MRSA and VRSA in ornamental birds and their interspecies transmission. In addition, these birds need to be under surveillance for early the detection of MRSA so that risk of their transmission to human could be minimized.

Acknowledgement

We sincerely acknowledge the Ministry of Science and Technology (MOST), Government of People's Republic of Bangladesh for providing the research grant (BS 300) to carry out the research.SBZ was supported by NST fellowship of MOST.

Ethical Statement

The experimental protocol of the present study was approved by the Institutional Ethical committee (AWEEC/BAU/2019(12)).

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