

Original Research Article

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Identification and Characterization of Methicillin Resistant Bacterial Isolates from Fish and Shellfish Collected from Cochin Backwaters, Kerala, India

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ABSTRACT

Keywords

Resistance, Antimicrobial, Emerging, Methicillin

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Resistance to antimicrobial agents among clinically important pathogens in the community and environment has compromised therapy and requires constant monitoring of emerging pathogens. Current investigation was aimed at determining the antimicrobial resistance pattern in bacteria isolated from public water body near a hospital where fishing is carried out and also from two fish farms which used the same water for aquaculture. In the present study methicillin resistance bacteria was recorded highest compared to other antibiotics and also this is the first report of the occurrence of clinical isolates such as *Klebsiella pneumoniae* and *Klebsiella oxytoca* with multi drug resistance in aquatic environment including in fish, suggesting the rapid spread of isolates and AMR genes between environments. The study revealed that imprudent use of antibiotics in hospital and release of the waste without prior treatment may have led to the dissemination of resistance from terrestrial to aquatic organism.

Introduction

The development and spread of antimicrobial resistance has emerged as a consequence of the selective pressure exerted by the increased antimicrobial usage in human medicine, veterinary medicine, animal production, fish production, agriculture and food technology. Horizontal transfer of genetic elements between bacteria is critical to the dissemination of resistance, particularly within a mixed bacterial population in the presence of antimicrobial drugs (Dermott *et al.*, 2003; Smillie *et al.*, 2011). Spread of antimicrobial resistance is not restricted by Phylogenetic,

geographic, or ecological barriers Thus the apparent overlap between the terrestrial and the aquatic environment, means that bacteria and the drug-resistance genes that they contain may be exchanged between these environments, implies a serious public health concern (Heuer *et al.*, 2009).

AMR in the context of human medicine has dominated the literature for a long time and numerous human commensals have developed resistance to one or more antimicrobials in clinical use. Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Enterococci* (VRE)

have also been found to have significant nosocomial ecology (Otter and French, 2010). Of other potential environmental reservoirs, wastewater has been identified as a possible source of exposure to MRSA in the community (Börjesson and Melin, 2009; Plano and Garza, 2011). The bacteria acquire resistance to Methicillin is most commonly mediated by *mecA* gene. The gene encodes for a penicillin-binding protein (PBP2a) which is expressed in the bacterial cell wall and has a low affinity for β -lactam antibiotics (Ito *et al.*, 2001)

Antimicrobials, resistant bacteria and resistance genes finally end up in the aquatic environment from terrestrial animals, sewage, hospital effluents and surface runoff. (Glynn and Bopp, 1998) found a correlation between resistant bacteria in rivers and urban water input.

It is apparent that aquatic organisms are continuously exposed to these resistant microorganisms present in water and in sediment, thereby undoubtedly influence the normal microflora.

To our knowledge, no studies have demonstrated the occurrence of Methicillin resistant bacteria other than MRSA associated with fish and shellfish of coastal waters. In the present study, we evaluated the occurrence of Methicillin resistant bacteria other than MRSA in fish and shellfish samples collected from a coastal water body adjacent to a known polluted source and aquaculture farms adjoining it.

Studies of Hossain and Aktaruzzaman, (2012) demonstrated multi drug resistant *Acinetobacter*, *Pseudomonas*, Enterobacteriaceae and phylogenetically distant bacteria such as members of alpha and beta proteobacteria in coastal water polluted with sewage water.

Materials and Methods

Isolation and identification of bacteria

The bacterial colonies that grew on the media were selected, purified and subjected to morphological and biochemical tests for identification. Tests carried out for identification included Gram's stain, catalase, oxidase, coagulase, motility, O-F, indole, gelatin hydrolysis, methyl-red, Voges-Proskauer, ONPG, lysine decarboxylase, citrate utilization, Triple Sugar Iron (TSI) agar, nitrate reduction and fermentation of sugars such as glucose, sucrose, lactose, mannitol, arabinose, sorbitol, inositol, fructose, mannose and rhamnose.

Antimicrobial susceptibility testing

Antibiotic resistance of bacterial isolates was determined by the disc diffusion method (Bauer *et al.*, 1996). The isolates were challenged with the following antibiotics: ampicillin (10 μ g), bacitracin (10 units), cefotaxime (30 μ g), cefpodoxime (10 μ g), cephalothin (30 μ g), co-trimoxazole (23 μ g), chloramphenicol (30 μ g), clindamycin (2 μ g), erythromycin (15 μ g), gentamicin (10 μ g), neomycin (30 μ g), ofloxacin (5 μ g), oxacillin (1 μ g), penicillin G (10 units), polymyxin B (300 μ g) and vancomycin (30 μ g). A bacterial suspension of overnight grown cultures was prepared and turbidity was adjusted to a 0.5 McFarland standard. A sterile cotton swab was used to inoculate the bacterial suspension on the surface of a Mueller Hinton Agar plate. Antibiotic impregnated disc (Hi-Media, Mumbai) were dispensed on the surface of inoculated agar plate and incubated overnight at 37 °C. The isolates were scored as susceptible, intermediate or resistant to a given antibiotic in duplicate by the inhibition zone diameter around the disc and according to the recommendations of the National Committee for Clinical Laboratory Standards

for antimicrobial susceptibility tests (NCCLS 2002).

PCR screening and characterization of *mecA* gene

The PCR used for detection of *mecA* gene using primers in (Table 1), according to Pereira and Lopes, (2009) with modification. The PCR was performed in a 50 µl reaction mixture containing 2.0 µl of template DNA, 1X assay buffer (10 mM Tris-HCl, pH 9.0, 1.5 mM MgCl₂, 50 mM KCl, 0.01% Gelatin), 100 µM of each of the four dNTP's, 10 picomoles of forward and reverse primers and 0.9 U of *Taq* DNA polymerase (Sigma, USA). PCR was performed using programmable thermocycler (ABI Veriti, USA). The optimized PCR programme for *mecA* gene consisted of initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min.

The amplified DNA fragments were sequenced with an automated ABI 3100 Genetic analyzer using fluorescent label dye terminators, by M/s Eurofins, Bangalore. The sequence information obtained in the present study was compared with all similar sequences available in the GenBank database. The Basic Local Alignment Search Tool (BLAST) was used to find out the sequence homology of *mecA* amplicons with the other *mecA* sequences available in the GenBank and the

Phylogenetic analyses were performed using MEGA version 3.1.

Primers used for PCR in the present study is given in Table 1.

Results and Discussion

In the present study a total of 56 bacteria were isolated, of which 32 from fish 12 from shrimp and 12 from clam. Among the 105 isolates recovered from the samples collected, (90.47%) tested positive for Methicillin resistance by disc diffusion. Out of the 95 isolates resistant to Methicillin as revealed by disc diffusion assay, 26 isolates, shown positive result for the presence of *mecA* gene (Table 2). Positive result showed a band at 535 bp using two primers internal to *mecA* gene (Fig. 1). Phylogenetic tree generated using the 535 bp of *mecA* gene of the bacteria of this study with other *mecA* genes of other MRSA on database after performing BLAST search and genetic alignment. Phylogeny showed that MRSA of this study is related from other MRSA of human or animal origin (Fig. 2) and BLAST algorithm showed 98% sequence similarity to *Staphylococcus aureus* Methicillin resistant protein (*MecRI*) and penicillin binding protein 2a genes (*mecA*) genes.

The presence of *mecA* gene among bacterial isolates is given in Table 2.

Table.1 Primers used for PCR in the present study

| Gene | Primer sequence (5' to 3') | Product size(bp) |
|----------------------------------|--|------------------|
| <i>mecA</i> F1 and <i>mecA</i> R | GGGATCATAGCGTCATTATTC AACGATTGTGACACGATAGCC | 535 bp |

Table.2 The presence of *mecA* gene among bacterial isolates

| S.No | Source of isolation | Isolates | <i>mecA</i> Gene |
|------|---------------------|------------------------------------|------------------|
| 1 | Fish | <i>Klebsiella oxytoca</i> | + |
| 2 | | <i>Klebsiella pneumoniae</i> | + |
| 3 | | <i>Streptococcus equi</i> | - |
| 4 | | <i>Voluribacter psittacidia</i> | + |
| 5 | | <i>Pseudomonas alcaligenens</i> | - |
| 6 | | <i>Enterococcus phoeniculicola</i> | - |
| 7 | | <i>Serratia antomophila</i> | - |
| 8 | | <i>Moraxella anatispestifer</i> | + |
| 9 | | <i>Edwarsiella tarda</i> | + |
| 10 | | <i>Enterobacter aerogenes</i> | + |
| 11 | Shrimp | <i>Acinetobacter johnsonii</i> | + |
| 12 | | <i>Klebsiella pneumoniae</i> | + |
| 13 | | <i>Klebsiella oxytoca</i> | + |
| 14 | | <i>Edwarsiella tarda</i> | + |
| 15 | | <i>Pseudomonas pertucinogena</i> | + |
| 16 | | <i>Acinetobacter johnsonii</i> | + |
| 17 | | <i>Klebsiella pneumoniae</i> | + |
| 18 | | <i>Klebsiella oxytoca</i> | + |
| 19 | Clam | <i>Edwarsiella tarda</i> | + |
| 20 | | <i>Voluribacter psittacidia</i> | + |
| 21 | | <i>Pseudomonas alcaligenens</i> | + |
| 22 | | <i>Enterococcus phoeniculicola</i> | + |
| 23 | | <i>Serratia antomophila</i> | + |
| 24 | | <i>Moraxella anatispestifer</i> | + |
| 25 | | <i>Voluribacter psittacidia</i> | + |
| 26 | | <i>Pseudomonas alcaligens</i> | + |

Fig.1

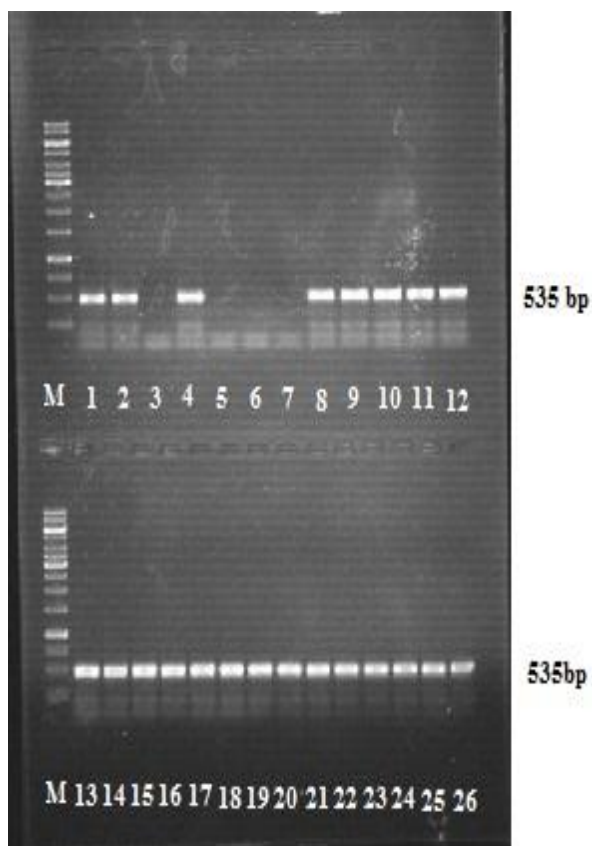


Fig.2

| Description | Max score | Total score | Query cover | E value | Ident | Accession |
|--|-----------|-------------|-------------|---------|-------|----------------------------|
| Staphylococcus aureus DNA_SCCmercury_SCCmec type III.1.1 (III A), complete sequence, strain OC3 | 985 | 985 | 100% | 0.0 | 98% | AB983237.1 |
| Staphylococcus aureus subsp. aureus strain Gv69, complete genome | 985 | 985 | 100% | 0.0 | 98% | CP009681.1 |
| Staphylococcus aureus strain XN108, complete genome | 985 | 985 | 100% | 0.0 | 98% | CP007447.1 |
| Staphylococcus haemolyticus DNA_cassette chromosomes, strain SH621 | 985 | 985 | 100% | 0.0 | 98% | AB478934.1 |
| Staphylococcus sciuri subsp. carnaticus GVGS2 SCCmec-mecC region, strain GVGS2 | 985 | 985 | 100% | 0.0 | 98% | HG515014.1 |
| Staphylococcus aureus subsp. aureus Z172, complete genome | 985 | 985 | 100% | 0.0 | 98% | CP008838.1 |
| Staphylococcus aureus Bmb9393, complete genome | 985 | 985 | 100% | 0.0 | 98% | CP005288.1 |
| Staphylococcus aureus CA-347, complete genome | 985 | 985 | 100% | 0.0 | 98% | CP006044.1 |
| Staphylococcus aureus subsp. aureus SCCmec staphylococcal cassette region, isolate CMFT532 | 985 | 985 | 100% | 0.0 | 98% | HF569114.1 |
| Staphylococcus aureus subsp. aureus SCCmec staphylococcal cassette region, isolate CMFT492 | 985 | 985 | 100% | 0.0 | 98% | HF569112.1 |
| Staphylococcus aureus subsp. aureus SCCmec staphylococcal cassette region, isolate CMFT489 | 985 | 985 | 100% | 0.0 | 98% | HF569111.1 |
| Staphylococcus aureus subsp. aureus SCCmec staphylococcal cassette region, isolate CMFT453 | 985 | 985 | 100% | 0.0 | 98% | HF569110.1 |
| Staphylococcus aureus subsp. aureus SCCmec staphylococcal cassette region, isolate CMFT352 | 985 | 985 | 100% | 0.0 | 98% | HF569107.1 |
| Staphylococcus aureus subsp. aureus SCCmec staphylococcal cassette region, isolate CMFT333 | 985 | 985 | 100% | 0.0 | 98% | HF569106.1 |
| Staphylococcus aureus subsp. aureus SCCmec staphylococcal cassette region, isolate CMFT2 | 985 | 985 | 100% | 0.0 | 98% | HF569101.1 |
| Staphylococcus aureus subsp. aureus SCCmec staphylococcal cassette region, isolate CMFT293 | 985 | 985 | 100% | 0.0 | 98% | HF569100.1 |
| Staphylococcus aureus subsp. aureus SCCmec staphylococcal cassette region, isolate CMFT151 | 985 | 985 | 100% | 0.0 | 98% | HF569095.1 |
| Staphylococcus aureus subsp. aureus SCCmec staphylococcal cassette region, isolate CMFT120 | 985 | 985 | 100% | 0.0 | 98% | HF569094.1 |
| Staphylococcus aureus strain 13101 methicillin-resistance protein (mecR1) and penicillin-binding protein 2a (mecA) genes, complete cds | 985 | 985 | 100% | 0.0 | 98% | JQ582126.1 |
| Staphylococcus aureus strain 4981 methicillin-resistance protein (mecR1) and penicillin-binding protein 2a (mecA) genes, complete cds | 985 | 985 | 100% | 0.0 | 98% | JQ582125.1 |
| Staphylococcus aureus strain 4977 methicillin-resistance protein (mecR1) and penicillin-binding protein 2a (mecA) genes, complete cds | 985 | 985 | 100% | 0.0 | 98% | JQ582124.1 |

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major problem in human clinical setting because it has a low affinity for all beta-lactam antimicrobials (penicillins, cephalosporins, carbapenems) and its widespread throughout the world since its emergence in the early 1960s (Jevons,1961). Recently, MRSA have emerged as a significant problem in veterinary medicine, including both animal and public health standpoints (Nishijima *et al.*, 2002; Chambers HF, Shabir *et al.*, 2010). Resistance of staphylococci to methicillin and all β -lactam antibiotics is associated with the low affinity of a penicillin-binding protein, PBP2a (Pierre *et al.*, 1990; Muhammad *et al.*, 2006), encoded by the *mecA* gene, which is located in the *mec* region and which is DNA of foreign origin (Matsushashi *et al.*, 1986). Acquisitions of Methicillin resistant determinants among the genome of other *staphylococcal* isolates and also association of MRSA with cultured fishes and marine mammals, (Soliman *et al.*, 2014) are well documented. Still the presence of these Methicillin resistant determinates among bacterial isolates other than MRSA is not well studied.

The present study demonstrated the presence of Methicillin resistance among bacterial isolated from fish and shellfishes.

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