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Determination of *mecA* expression and other resistance mechanisms in methicillin-resistant *Staphylococcus aureus* isolated from *Oreochromis niloticus* (Nile tilapia)

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Methicillin-resistant *Staphylococcus aureus* is a major cause of infection worldwide. Production of β-lactamases and penicillin-binding protein 2a are the two main mechanisms of resistance in *S. aureus.* The aim of this work was to study the mechanisms of resistance produced by the *S. aureus* strain isolated from *Oreochromis niloticus* (Nile tilapia) during an outbreak. β-Lactamases production was detected by iodometric and clover leaf techniques. The induction of *mecA* gene was done using oxacillin and the gene expression was detected by real time reverse transcription-polymerase chain reaction in the induced isolate and compared with the non-induced one. Also, the studied *mecA* gene was sequenced to check the similarity between the gene of the tested isolate and the published *mecA* genes. Results showed that this isolate produces β-lactamase and *mecA* expression was seven times increased in the case of oxacillin induction. Sequencing results showed 99% identity between the studied gene and the published reference genes. Extensive use of antibiotics in fish farms resulted in the emergence of multidrug resistant staphylococci and this resistance may be induced by the continuous use of some antibiotics.

Key words: Methicillin-resistant *Staphylococcus aureus* (MRSA), *mecA* gene, induction, gene expression.

INTRODUCTION

Outbreaks of bacterial diseases in fish remains one of the most significant limiting factors affecting fish culture worldwide (Gisain et al., 2013) and are among the most important causes of economic losses in cultured tilapia

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(Martins et al., 2008). Staphylococci are among the most widespread opportunist pathogenic bacteria. It has been reported as the causative agent of eye disease on Silver carp (Shah and Tyagi, 1986) and *Channa marulius* (Kumaraiah et al., 1977). Also, methicillin-resistant *Staphylococcus aureus* (MRSA) has been isolated from apparently healthy *Oreochromis niloticus* (Nile tilapia) (Atyah et al., 2010).

MRSA is a major cause of infection worldwide (Rudkin et al., 2014). The emergence and subsequent spread of MRSA from the 1960s to the present, has created clinical difficulties for nosocomial treatment on a global scale (Fuda et al., 2005). Staphylococci have two mechanisms for resistance to beta-lactam antibiotics. One is the production of beta-lactamases, which are enzymes that hydrolytically destroy beta-lactams. The other is the expression of penicillin-binding protein 2a (PBP 2a), which is not susceptible to inhibition by beta-lactam antibiotics. Strains of *S. aureus* exhibiting either beta-lactamase or PBP 2a-directed resistance (or both) have established a considerable ecological niche among pathogenic bacteria (Fuda et al., 2005).

The major mechanism of methicillin resistance in *S. aureus* is the acquisition and expression of *mecA* gene that encodes penicillin-binding protein 2a (PBP2a) (Shang et al., 2010) which has low affinity to beta-lactam antibiotics (Hussain et al., 2000). Regulation of *mecA* expression is controlled by its own regulators, *mecR1* and *mecI*. In the absence of β-lactam antibiotics, *mecA* transcription is repressed by *MecI* bound to its promoter region. Detection of β-lactams by the sensory domains in *MecR1* removes the repression of *mecA* transcription by *MecI*, which leads to *mecA* transcription, PBP2a translation and the expression of methicillin resistance (Rudkin et al., 2014).

Beta-lactam compounds such as penicillin continues to be one of the most frequently used drugs in veterinary medicine (Robles et al., 2014). And it is recommended as the first choice for bacteria that are inherently sensitive to it. In contrast to human isolates, the prevalence of penicillin resistance in staphylococci causing animal diseases can be relatively low and is most commonly due to the production of β –lactamase (Pitkälä et al., 2007).

The aim of this study was to detect the mechanisms of resistance to beta lactam antibiotics in the MRSA isolate causing the outbreak of infection in Nile Tilapia, and to determine the effect of oxacillin on *mecA* expression in the tested MRSA isolate.

MATERIALS AND METHODS

Bacterial isolate

S. aureus isolate was obtained from the kidney of naturally infected Nile Tilapia during an outbreak in Kafr-Elsheikh governorate. Its identification was done by Gram staining, catalase, coagulase tests, cultivation on mannitol salt agar and confirmed by API Staph system.

Antimicrobial susceptibility testing

Susceptibility testing was determined by the disc diffusion method according to Clinical and Laboratory Standards Institute (Wayne 2011) guidelines for 17 antibiotics: chloramphenicol (C), lincomycin (L), cepheadine (CE), gentamicin (G), doxycycline (DO), norfloxacin (NOR), streptomycin (S), apramycin (APR), ampicillin (AMP), ciprofloxacin (CIP), cefoxitine (FOX), oxacillin(OX), amoxicillin-clavulanic acid (AMC), cefotaxime (CTX), ceftriaxone (CRO), ceftazidime (CAZ), cefepime (FEP).

Detection of β-lactamase production by the iodometeric method

Penicillin solution was dispensed in 0.5 ml volume in small test tubes. Test bacteria were removed with a loop from an overnight culture on solid medium and suspended in the Penicillin solution to give a density of at least 10^4 CFU/ml. After one hour at room temperature, two drops of starch indicator was added to the suspension, followed by one drop of Iodine reagent. Positive reaction was indicated by the disappearance of blue color immediately. Persistence of blue color for longer than 10 min constituted a negative test (Miles and Amyes, 1996).

Clover leaf technique

This method was done according to Parvathi and Appala (2000) with some modifications where a Mueller-Hinton agar plate was swabbed with a culture of β lactamase nonproducing strain of *S. aureus* ATCC 25923. An amoxicillin disc (10 units) was placed in the centre of the plate and the test strain was heavily streaked radially outward from the disc to produce growth about 0.25 cm wide. The plate was incubated at 37°C for 18 h and the examined for the presence of clover leaf pattern.

If the strains produced β-lactamase, the zone produced by the β lactamase nonproducing strain was inhibited where the zones of growth of ATCC strain and test strains coincided thus giving rise to a clover leaf pattern. If the test strains did not produce β-lactamase, no clover leaf pattern was produced.

Induction of *mecA* expression by using oxacillin

Induction of *mecA* expression was made according to Hussain et al. (2000) with slight modifications where the bacteria were swabbed on the surface of Mueller-Hinton agar and a disc with 1 µg of oxacillin was placed in the main inoculums. After 18 h incubation, the growth around the disc was used to perform the quantitative reverse transcription polymerase chain reaction (RT-qPCR). RT-qPCR was performed on both the induced and non-induced *S. aureus*, and then the results were compared.

Gene expression by RT-qPCR

RT-qPCR was performed according to method described by Shang et al. (2010) by using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA). 100 ng of RNA were used in each sample, and MyGenie 32 Thermal Block (BIONEER) was used to perform RT-PCRs.

Real time PCR was performed using SYBR® Green PCR Master Mix (Applied Biosystems, USA) and Stratagene Mx3000P QPCR system (Agilent technologies). The primers used are shown in Table 1.

Table 1. Primers used for gene expression real time PCR (Shang et al., 2010).

Primer	Primer sequence (5–3)	Target gene	Amplicon size (bp)
Mec F	CTCAGGTACTGCTATCCACC	*MecA*	152
Mec R	GGAACTTGTTGAGCAGAGG		
CON F	CCAGCAGCCGCGGTAAT	16S RNA	100
CON R	CGCGCTTTACGCCCAATA		

Sequencing of *mecA* gene

In order to detect the identity of the *mecA* gene of the tested isolate and to search for the presence of mutations that may affect the gene, sequencing of *mecA* gene was performed. The PCR product was purified using PureLink PCR purification kit (Invitrogen Life Technologies, Carlsbad, USA). Automated sequencing reactions were performed with the BigDye terminator cycle sequencing kit and an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster city, USA) using the same primers that were used in the amplification of the *mecA* gene.

Phylogenic analysis

The derived sequence was aligned and compared with those of published reference *S. aureus* strains in the GeneBank using the National Center for Biotechnology Information's BLAST server and the software package (BioEdit v 7.2.5) for multiple sequences alignment and phylogenetic tree construction.

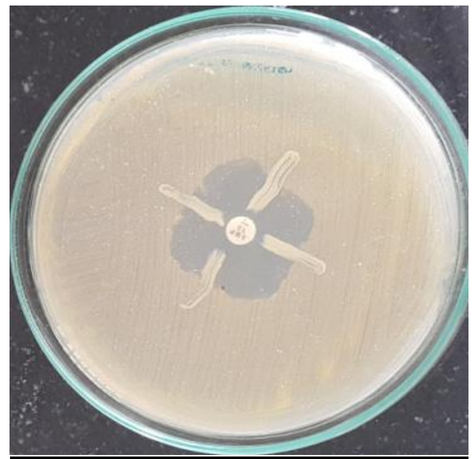

Figure 1. Clover leaf technique showing β lactamase positive result.

RESULTS

The examined isolate was found to be Gram-positive, catalase and coagulase positive. Using API staph system, the isolate was confirmed to be *S. aureus*.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing showed that the bacteria are resistant to all β-lactam antibiotics including ampicillin, cephradine, cefotaxime, ceftriaxone, ceftazidime and cefepime. It was also found to be resistant to oxacillin which indicates methicillin resistance. But they are sensitive to gentamicin, streptomycin, lincomycin, doxycycline, ciprofloxacin, norfloxacin and chloramphenicol.

Beta-lactamase production

The *S. aureus* isolate was found to be β-lactamase producer by the iodometric method where disappearance of the blue color was observed and this was confirmed by the clover leaf method where the zone produced by the control strain was inhibited and the zones of growth of the control strain and test strain coincides thus giving rise to a clover leaf pattern as illustrated in Figure 1.

Expression of *mecA* gene

It was found that oxacillin induced *mecA* expression in the tested MRSA isolate with increase in the expression of 8 folds as compared to the non-induced isolate.

Phylogeny of *mecA* gene

Multiple sequence alignment and phylogenic analysis of *mecA* gene indicated the great similarity (99% identity) between the tested gene sequence and the other *S. aureus* strains published in the Gene bank. This is also shown in the phylogenetic tree (Figure 2).

DISCUSSION

S. aureus is a major human pathogen and is resistant to most commercially available antibiotics. The antibiotic resistance crisis may be attributed to the overuse and misuse of antibiotics (Ventola, 2015). Because of the

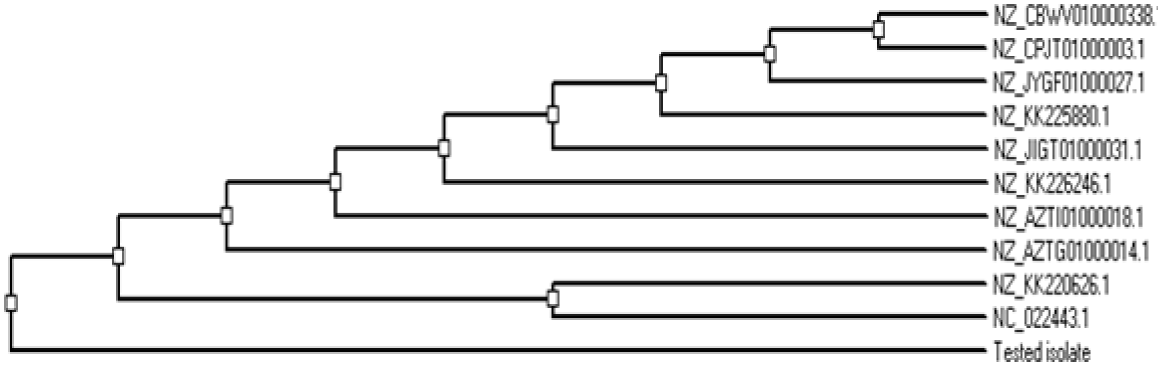

Figure 2. Phylogenetic tree of the *mecA* gene of the tested and reference *S. aureus* strains generated by the software package (BioEdit).

ability of staphylococci to change over time, the MRSA will continue to be a problem in the future. Production of β-lactamases and the expression of penicillin-binding protein 2a (PBP 2a) cause a tremendous problem in medicine because these render *S. aureus* to be resistant to all β-lactam antibiotics (penicillins, cephalosporins and carbapenems) (Lowy, 2003).

In this work, β-lactamase production in *S. aureus* strain isolated from Nile Tilapia fish was studied. The results of antimicrobial susceptibility test showed that the bacteria are resistant to all β-lactam antibiotics and oxacillin. For β-lactamase production, different phenotypic diagnostic methods that are specific and reliable such as the clover leaf and iodometric methods were used (Robles et al., 2014). The iodometric method was considered as an accurate method (Devapriya et al., 2013) while, the clover leaf method was considered as a reliable method for investigating β-lactamase production in staphylococci (Bergan et al., 1997). Whereas Pitkala et al. (2007) estimated that it is more useful in research than in routine use.

Concurrent exposure to antimicrobials may reduce susceptibility to antimicrobial drugs in the major human pathogen, *S. aureus* (Haaber et al., 2015). Here, methicillin resistance and the role of oxacilin in *mecA* gene induction was studied which shows the risk of misuse of antibiotics in increasing the virulence and resistance of staphylococci. Expression of *mecA* and PBP2a gene is known to be inducible by many β-lactam antibiotics with oxacillin being a more potent inducer than methicillin in some strains (Rudkin et al., 2014; Shang et al., 2010). These findings were similar to what is found in this work where there was about 8 folds increase in *mecA* gene expression after subjecting the isolate to oxacillin. The induction of *mecA* is strain dependant as it ranges from 2 to 50 fold increase as reported by Shang et al. (2010).

Cell wall-active antibiotics such as oxacillin, cause induction of a locus in *S. aureus* that leads to elevated synthesis of two methionine sulfoxide reductases (MsrA1 and MsrB). These enzymes reduce methionine sulfoxide and maintain protein integrity and function against oxidative stress. These two proteins have also been shown to have potential roles in bacterial virulence (Singh et al., 2015). There are also a number of studies that have shown that sub-inhibitory concentrations of oxacillin increase rather than decrease staphylococcal virulence by increasing the transcription of toxin genes such as alpha-toxin and Panton-Valentine leucocidin (PVL) in *S. aureus* strains. Oxacillin also induced an overall increase in exoprotein expression levels by MRSA isolates, including alpha-toxin and PVL, revealing that oxacillin has pleiotropic effects on *S. aureus* strains, altering their toxin expression profile (Rudkin et al., 2014).

Findings of this study show that fish play a role in transmission of methicillin-resistant *S. aureus* infection. Also, in concordance with other studies, subjecting the present study MRSA isolate to oxacillin increases the expression levels of *mecA* gene by eight folds.

Results of *mecA* sequencing showed that there is 99% identity between the gene of the tested isolate and those of the published reference strains; this indicated that this fish pathogen may be a source of infection to human who handle them or use them as food which may lead to intoxication. Finally it can be concluded that the misuse of antibiotics may result in the emergence of highly resistant bacteria which may be a health hazard to fish and may also be transmitted to humans.

Conflict of Interests

The authors have not declared any conflict of interests.

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