ORIGINAL ARTICLE

Evaluation of *bla* SHV, *bla* TEM and *bla* OXA encoding Clinical Isolates from Chronic Tonsillitis using Phenotypic and Molecular Technique: First report from Pakistan

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Author`s Contribution

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ABSTRACT

Objective: To evaluate *bla* SHV, *bla* TEM and *bla* OXA encoding clinical isolates in chronic tonsillitis using phenotypic and molecular techniques.

Place and Duration: The study was conducted in Department of Microbiology and Molecular Genetics, University of Punjab, Lahore from August 2016 to August 2017.

Methodology: Sample processing, identification and characterization of isolates was done by using (CLSI, 2016) criteria. Antibiotic susceptibility testing by using disc diffusion assay and biofilm forming ability was analyzed by ring test and slime production test. Combination disc test was used for phenotypic detection of antibiotic resistance genes. Multiplex-PCR assay was used to check the presence of *bla* SHV, *bla* TEM and *bla* OXA genes. 16S rRNA sequencing and phylogenetic analysis was performed.

Results: Here, variable resistance pattern was observed against applied antibiotics. 100 % resistance towards azotreonam and penicillin was observed. While 60-85 % resistances were observed against cephalosporins. Biofilm formation increased with the passage of time. 77% strains indicated positive combination disc test. Multiplex-PCR indicated 60% strains harbored tested genes. 40 % *bla* SHV genes, 30 % *bla* TEM genes and 60% *bla* OXA genes were observed among selected isolates. GenBank Accession number obtained for *Klebsiella pneumoniae was* KY810693 and for *S. aureus* was KY810692.

Conclusion: In conclusion, *K. pneumoniae* and *S. aureus* came out to be common causative agents of tonsillitis in the current study. Resistance towards multiple classes of antibiotics and strong biofilms of these micro-organisms explain the chronicity and recurrent nature of the infection. *bla* OXA genes were frequent among genes tested.

Keywords: tonsillitis, biofilm, antibiotic resistance genes, *bla* SHV, *bla* TEM, *bla* SHV.

Introduction

Tonsillitis is characterized by inflamed tonsils, usually with difficulties in terms of swallowing and breathing at the night.[1-3] Tonsils have gained scientist's attention much in the past decade because of their part in immune defenses as well as in pathogenesis of some diseases

plus prion infections.[4, 5] The key viral antigens of tonsillar infection are para influenza virus, adenovirus, influenza virus, rhinovirus, and the family of herpes-viruses.[6] *Streptococcus pyogenes* and *Haemophilus influenzae* are the most popular bacterial pathogen of acute tonsillar disease[7,8] The most prevalent pathogenic bacterial species in recurrent chronic tonsillitis is *Staphylococcus aureus,* while in peritonsillar abcess *Streptococcus pyogenes*, *Haemophilus influenza* and *Haemophilus parainfluenzae.*[9.]

Antibiotics have proved very active in the treatment of tonsillitis for several decades. It is suggested that the duration of treatment should be on a selective basis, using a ten-day, or short antibiotic course, according to circumstances.[10] The immune system of host effecting the formation of bacterial biofilm on the tissue of tonsils and this response to the infections are of importance.[11] Biofilms offer a shielding environment against immune reactions as well as against antibiotic actions. This may frequently relate to recurrent or chronic infections of bacteria. The presence of bacterial biofilms in tissues like adenoid and tonsils tissue may support to describe the hard eradication of bacterial species, involved in chronic infective processes. [12, 13.]

Multidrug-Resistant (MDR) phenotype is due to the production of certain enzymes which inhibit the functions of antibiotics and hence results in failure of therapy.[14] These enzymes are encoded by multiple genes residing on the bacterial genome. Most of such enzymes are encoded by genes residing on plasmids which can easily be transferred between bacteria. *bla* SHV, *bla* TEM and *bla* OXA genes are known for antibiotic-resistance in *Klebsiella* spp, *E. coli* and some strains of *S. aureus*. Nowadays, these genes have spread among all clinically important bacteria by horizontal gene transfer by utilization of plasmids. *bla* CTX-M, *bla* SHV, *bla* TEM and *bla* OXA are most frequently associated with cephalosporin resistance in South-East Asia.[15, 16]

Here, we evaluated *bla* SHV, *bla* TEM and *bla* OXA encoding clinical isolates in chronic tonsillitis using phenotypic and molecular techniques.

Methodology

Bacterial Isolates: The present study was conducted in Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore Pakistan from August 2016 to August 2017. Ethical Board of Citilab and Research Centre approved the study with Reference # 26-16/CLRC/24th. Post-tonsillectomy Tonsillitis specimens were processed according to standard operating procedures. A total of 13 strains were an isolated and biochemical characterization of isolated strains was carried out by using manual method as well as API-20E strips. The isolates were preserved in glycerol stocks and stored at -20°C.

Antibiotic Susceptibility Test (AST): Disc diffusion assay was used to formulate antibiogram profile of isolated strains [17]. A panel of antibiotics recommended by Clinical Laboratory Standard Institute (CLSI, 2017) was used that has previously been used in another study.[18] Multiple Antibiotic Resistance (MAR) value was calculated by method of Krumperman.[19]

Biofilm Formation Test: Biofilm formation of strains was determined by using Ring test [20] and slime production was checked by using Congo Red test.[20, 21]

Phenotypic Detection Tests for antibiotic resistance genes (ARG): Ability of isolated bacteria to produce certain enzymes was checked by using combined Disc test in which cefotaxime (30μg) and ceftazidime (30 μg) discs were used alone as well as in combination with clavunate (10 μg). If the difference of zone sizes between discs alone and in combination with clavunate was greater than 5 it was denoted as positive.[18]

Molecular Detection Tests for ESBLs: Multiplex-PCR was used through designing a colony PCR for the *bla* SHV, *bla* TEM and *bla* OXA genes. Here, 2 μl DNA was used in 25 μl PCR master mixture containing 10x PCR buffer, 1.5 Mm $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (dNTPs), 20 pmol of three primer sets [22] and 2.5 U of *Taq* polymerase (Thermo scientific, Germany). PCR amplification conditions were: Initial step of denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95° C for 1 min then annealing at 56° C for 1:30 min, extension at 72 C for 1 min and the final extension was done at 72° C for 10 min. PCR amplification primers for genes are: *bla* SHV (Forward: 5′AGGATTGACTGCCTTTTTG3′, Reverse: 5′ ATTTGCTGATTTCGCTCG3′):*bla* TEM (Forward: 5′ATCAGCATAAACCAGC3′,Reverse:5′CCCCGAAGAACT3′

) and *bla* OXA (Forward:5′ ATATCTCTACTGTTGCATCTCC3′,Reverse:5′AAACCCTTC AAACCATCC3′)[23]. After amplification, PCR products were run on 1% agarose gel which was further visualized on UV-trans illuminator.

16S rRNA Sequencing and Phylogenetic Analysis: 16S rRNA sequencing for strain confirmation was performed using commercial sequence services of Advance Bioscience International, Pakistan in collaboration with 1st Base, Malaysia. NCBI-BLAST (www.ncbi.nlm.nih.gov/BLAST/) was used to check the similarity of obtained sequences with already submitted sequences.[24] GenBank Accession numbers were obtained by submitting our refined sequences. MEGA 6.0 software was used for sequence alignment and phylogenetic analysis.[25]

Results

Bacterial Isolates and Antibiotic Susceptibility Test: Colonies with characteristics growth pattern were selected on MacConkey's agar plate, beta hemolytic and gamma hemolytic colonies from blood agar were selected. Here, 38 % strains were gram positive cocci (*Staphylococcus aureus*) and 15 % were gram negative bacilli (*Klebsiella* spp). Most of the strains were resistant to applied antibiotics with a few exceptions. All strains were resistant to ATM while 69 % strains were resistant to TPZ, 69 % were resistant to ZOX, 15 % were resistant to CEP, 38 % were resistant to CE, 23 % were resistant to CTX, 53 % were resistant to CXM, 15 % were resistant to CRO, 46 % were resistant to FOX, 69 % were resistant to CXA, 38 % were resistant to CN, only one strain was resistant to AK, 53 % were resistant to B, 15 % were resistant to CIP, 61 % were resistant to AM, 53 % were resistant to P, 84 % were resistant to AMC, and all strains were resistant to CX. Resistance pattern was ATM and CX 100 % >AMC 84 % > TPZ 69 % =ZOX 69 % =CXA 69 % > AM 61 % > CXM 53 % =B 53 % =P 53 % > FOX 46 % > CE 38 % =CN 38 % >CTX 23 % >CEP 15 % =CRO 15 % = CIP 15 % (Figure 1).

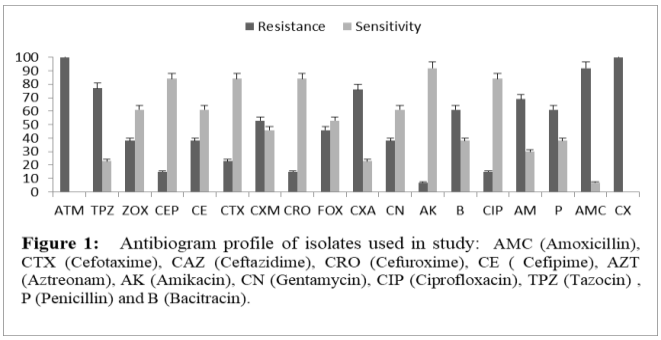

Figure 1: Antibiogram profile of isolates used in study: AMC (Amoxicillin), CTX (Cefotaxime), CAZ (Ceftazidime), CRO (Cefuroxime), CE (Cefipime), AZT (Aztreonam), AK (Amikacin), CN (Gentamycin), CIP (Ciprofloxacin), TPZ (Tazocin) , P (Penicillin) and B (Bacitracin).

Biofilm Formation Test: Biofilm formation increased with time. Biofilm formation by strain *Staphylococcus aureus* (BMC1) and *Klebsiella pneumoniae* (BMT1) were increased with the passage of time therefore these strains showed multidrug-resistance phenotype (Figure 2). Strain BMC1 and BMT1 showed colonies with black centers indicating slime production in presence and absence of glucose in the medium.

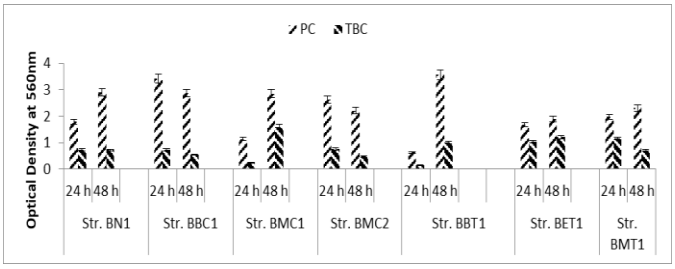

Figure 2: Ring test of strains: Selective strains were tested for biofilm formation and were monitored for over of period of time to check change in biofilms over the period of time. Here, PC and TBC were two tonsilitis samples.

Phenotypic Detection Tests for ESBLs: 77 % (10/13) isolates indicated positive combination disc test. While 23 % (3/13) isolates remained non-determined by this technique.

Molecular Analysis of ESBLs genes: Multiplex-PCR indicated 60 % (6/10) strains harbored tested genes. Here, 40 % *bla* SHV genes, 30 % *bla* TEM genes and 60 % *bla* OXA genes were observed among selected isolates. While all three genes were present in 20% isolates (Figure 3A and B).

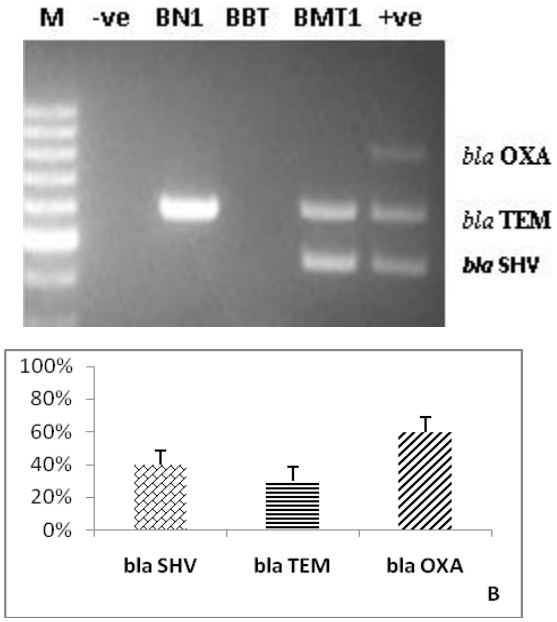

Figure 3: A) 1% Agarose gel electrophoresis of Multiplex-PCR products and B) Molecular Analysis of *bla* SHV, *bla* TEM and *bla* OXA genes.

16S rRNA Sequencing and Phylogenetic Analysis: MEGA 6.0 software was used for sequence alignment. GenBank Accession number obtained for *Klebsiella pneumoniae was* KY810693 and for *S. aureus* it was KY810692. Boots strap value for *Klebsiella pneumoniae* is 0.0005 and of *S. aureus* is 0.0002. Horizontal lines showed branches and indicated evolutionary lineages that changed over time (Figure 4).

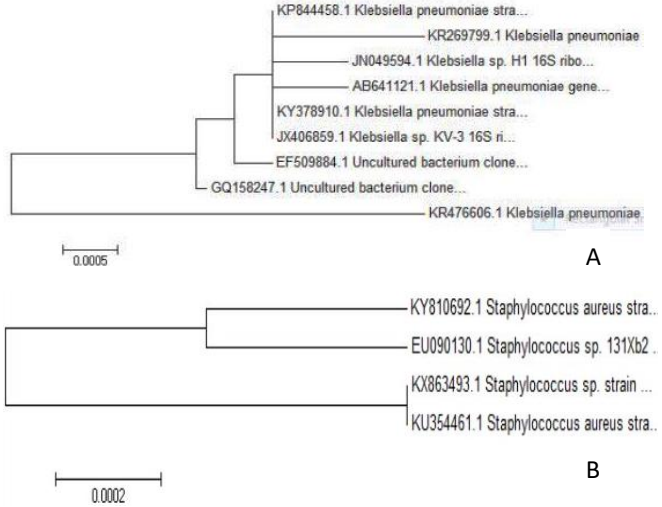

Figure 4: Phylogenetic tree of isolated strains. (A) *Klebsiella pneumoniae* and (B) *Staphylococcus aureus*

Discussion

Recurrent tonsillitis is one of the important respiratory tract infections. Its diagnosis and treatment is important especially for children as 12-15% of children all the time have *Streptococcus pyogenes* in their tonsils.[15, 26] Involvement of gram positive and gram negative bacteria have previously been reported. Here, we report 38 % strains were gram positive cocci (*Staphylococcus aureus*) and 15 % were gram negative bacilli (*Klebsiella* spp). Similar finding were observed in throat swabs of patient with tonsils in the University of Maryland Medical Center where *Klebsiella pneumoniae* was isolated.[27] Multidrug-resistant (MDR) *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus viridians*, *Neisseria spp*. and *Streptococcus pyogenes* were isolated from tonsillitis in other parts of world. [4, 9, 26]

Amoxicillin and amoxicillin-clavunate (AMC) are being used to treat tonsillitis since long time. Resistance profile indicated that isolates were highly resistant towards AMC and group of cephalosporins. Such type of resistance profile has already been observed in a report from University of Benin, Nigeria where high resistance was observed towards these antibiotics [28]. Frequent utilization of AMC might cause such high resistance in bacteria. Isolates showed promising susceptibilities towards ciprofloxacin, amikacin and gentamycin. These results are in accordance with the recurrent tonsillitis samples where similar susceptibility patterns were observed.[28, 29] Biofilms have proved to be important contributors towards chronic tonsillitis.[30] Biofilm formation was observed to be increased in prolonged infections which complicates the situation and hinders the therapy.[31]

Molecular analysis of antibiotic-resistance genes indicates involvement of multiple genes to develop resistant phenotype. Moreover, co-resistance of genes may indicate the severity of such infections which might not be treated with traditional antibiotics. Yigit et al., also reported the presence of *bla* SHV and *bla* TEM genes in *K. pneumoniae* by specific PCRs.[32] We have observed high frequency of *bla* OXA genes compared to *bla* TEM and *bla* SHV genes. Sugumar et al. reported similar findings where *bla* OXA-1 genes were prevalent compared to other β-lactam genes screened.[33]

Conclusion

In conclusion, *K. pneumoniae* and *S. aureus* came out to be causative agents of tonsillitis in current study. Resistance towards multiple classes of antibiotics and strong biofilms of

these micro-organisms explain the chronicity and recurrent nature of the infection. *bla* OXA genes were frequent among genes tested. This is a first report from Pakistan which indicates a scientific challenge for the community and health care settings. Based on this report further research plans will be formulated to eradicate this dilemma from society.

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