INVESTIGATION AND ANTIBIOTIC SUSCEPTIBILITY OF STREPTOCOCCUS PNEUMONIAE ISOLATED FROM PATIENTS WITH OTITIS

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ABSTRACT: Background and Aims: Streptococcus pneumoniae is the most frequent cause of bacterial meningitis, community-acquired bacterial pneumonia and acute otitis media (AOM). Eighty percent of children have at least one episode by three years of age. This study was conducted to determine the frequency of S. pneumoniae and also antibiotic susceptibility patterns of this microorganism in patients with acute middle ear infections. Materials and Methods: During a period of 10 month, 60 middle ear discharge specimens were collected from patients with acute otitis media in Amir Alam Hospital. Specimens were assessed for Streptococcus pneumoniae by microscopic examination and culture. PCR technique was used to identify genes lytA, For definitive identification of Streptococcus pneumoniae .To identify the encapsulated strains of the PCR technique was used to identify genes cpsA. The antibiotic susceptibility test was done by Kirby-Bauer disk diffusion method according to CLSI (clinical and laboratory standard institiute) criteria. Results: Out of 60 tested specimens, 8 isolates were confirmed as Streptococcus pneumonia by culture method.. The sensitivity of isolated Streptococcus pneumoniae to different antibiotics included Tetracycline (56%), Erythromycin (67%), Vancomycin (22%), Chloramphenicol (56%), and Amoxicillin clavulanate (22%),Rifampine (44.4%), Ciprofloxacin (56%), levofoxacin (0%) Trimethoprim+Sulfa (77.8%). 14 samples were positive for lytA.after the PCR of 60 middle ear efusion, and all having a capsule. Conclussion: Continuous surveillance in order to determine full picture of antibiotic susceptibility in Streptococcus pneumonia isolated from patients with AOM is necessary.The present study exhibited that the rate of resistance to antibiotics is increasing. Third-generation cephalosporins had good activity against S.pneumonia isolated from acute otitis infections.

Keywords: Antimicrobial Susceptibility, Acute Otitis Media, Streptococcus Pneumonia.

INTRODUCTION
Otitis media is one of the most common childhood diseases, Otitis media can caused by vi¬ruses, allergy, bacteria and their products, and the dysfunc¬tion of the Eustachian tube[1].Otitis media with effusion (OME) and acute otitis media (AOM) are the two major sub-classifications of otitis media[2].OME is characterized by the presence of middle ear fluid without acute infection[3] Streptococcus pneumonia, Haemophilus influenzae, and Moraxella catarrhalis have been reported as the most common pathogenic microorganisms in Otitis media. Streptococcus pneumoniae is one of the bacterial causes of community-acquired pneumonia, acute otitis media (AOM), bacteraemia, sinusitis and meningitis, particularly in children and elderly people.
The bacteria permeate the nasal cavity and attach to the nasopharyngeal epithelial cells and spread to other organs, such as the ears, sinuses, and then potentially penetrate the mucosal barrier to enter the bloodstream and/or cross the blood–brain barrier to cause meningitis. This organism was uniformly susceptible to penicillin before 1967. The first clinical isolate of S. pneumoniae not susceptible to penicillin was reported in 1986 [4]. Pneumococcal resistance to antimicrobial drugs was first reported in the mid-1960s [5, 6]. Non-susceptibility to penicillin in S. pneumoniae was first described in Australia in 1967, and later in (1974) in New Guinea, and in (1977) in South Africa, and Spain (1979) [7, 8]. Reduced susceptibility to penicillin in S. pneumoniae is associated with target alterations in penicillin-binding proteins (PBPs), leading to decreased affinities for the antibiotics [9]. The aim of this study is investigation and antibiotic susceptibility of Streptococcus pneumoniae isolated from patients with otitis.

MATERIALS AND METHODS

Study subject and sample collection

In this descriptive study, specimens of middle ear effusions were obtained from 60 patients, who were candidates for myringotomy in the department of otolaryngology of Amir Alam hospitals of Tehran University of Medical Sciences during 10 months from March 2014 to February 2015. All the patients had middle ear effusion for more than 3 months and none of them had any antibiotic therapy 2 weeks before and at the time of surgery. The external ear canal was disinfected with povidone–iodine and then washed with sterile normal saline for removing the antiseptic agents. Then, middle ear fluid was aspirated into a Juhn-Tym-Tap collector [10]. Within 2 h after sampling, specimens of middle ear effusions were sent to the Laboratory of Microbiology Department of Shahid Beheshti University.

Isolation of bacteria

Specimens were inoculated to blood agar under aerobic conditions with 5% CO2 at 35 °C for 18–24 h [11, 12]. Alpha-hemolysis, bile solubility tests and optochin sensitivity was used to identify S. pneumoniae isolates. The STGG medium has been used for storage of S. pneumonia [13]. In brief, mix 2.0 g of skim milk powder, 0.5 g of tryptone soy broth powder, 0.5 g of glucose, and 10 ml of glycerol and dissolve in 100 ml of distilled water. The STGG medium should be autoclaved before use: dispense 1 ml of STGG medium into 1.5 ml screw-capped vials and autoclave for 10 min at 121 °C. STGG vials can be stored frozen at −20 °C (or colder) or refrigerated until use.

Antimicrobial sensitivity

The disk agar diffusion (DAD) method was used to determine the susceptibility patterns of isolated, according to the CLSI (Clinical and Laboratory Standards Institute) guidelines [3]. The antibiotics tested were as follows: amoxicillin 25 μg, amoxicillin/clavulanate 30 μg, erythromycin 15 μg, chloramphenicol 30 μg, tetracycline 30 μg, cotrimoxazole 25 μg, ciprofloxacin 30 μg, levofloxacin 5 μg, rifampicin 5 μg, and vancomycin 30 μg (Mast Group, Merseyside, UK).

MIC test

Minimal inhibitory concentrations (MICs) for penicillin, ceftriaxone were determined by micro broth dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2010). The quality control strain was S. pneumoniae ATCC 49619. The results were interpreted according to CLSI guidelines for breakpoints.

2.4. Extraction of DNA from bacterial cultures.

DNA was extracted by Phenol–chloroform [3].

PCR

PCR amplification of lytA and cpsA genes was done with specific primers according to Table 1 [14]. Prepare materials and Regulation of Thermocycler system for detection lytA and cpsA genes are shown in Table 2 and Table 3 [15, 16].
Table 1-Primer Characteristics.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lytA</td>
<td>F-5'ACGCAATCTAGCAGATGAAGCA3</td>
<td>76 bp</td>
</tr>
<tr>
<td></td>
<td>R-5-TCGTGCCTTTAAATTCCAGCT-3</td>
<td></td>
</tr>
<tr>
<td>cpsA</td>
<td>F-5-GCAGTACAGCAGTTGGGACTGACC-3</td>
<td>160 bp</td>
</tr>
<tr>
<td></td>
<td>R-5-GAATATTTCTATTACAGTCCCAGTC-3</td>
<td></td>
</tr>
</tbody>
</table>

Table 2-Prepare materials for PCR the lytA and cpsA genes.

<table>
<thead>
<tr>
<th>Material</th>
<th>volume</th>
<th>concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDW</td>
<td>8.5</td>
<td>-</td>
</tr>
<tr>
<td>Primer forward</td>
<td>1</td>
<td>20 pico mol</td>
</tr>
<tr>
<td>Primer reverse</td>
<td>1</td>
<td>20 pico mol</td>
</tr>
<tr>
<td>Master Mix</td>
<td>12.5</td>
<td>1.5 unit</td>
</tr>
<tr>
<td>DNA template</td>
<td>2</td>
<td>20ng</td>
</tr>
<tr>
<td>Total volume</td>
<td>25</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3-Regulation of Thermocycler System for Detection LytA and CpsA Genes

<table>
<thead>
<tr>
<th></th>
<th>cpsA</th>
<th>lytA</th>
<th>gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cycles</td>
<td>time</td>
<td>Temperature</td>
<td>Number of cycles</td>
</tr>
<tr>
<td>1</td>
<td>5 min</td>
<td>95 ºc</td>
<td>1</td>
</tr>
<tr>
<td>30</td>
<td>1 min</td>
<td>95 ºc</td>
<td>30</td>
</tr>
<tr>
<td>1</td>
<td>63 ºc</td>
<td>45s</td>
<td>45s</td>
</tr>
<tr>
<td>1</td>
<td>72 ºc</td>
<td>30s</td>
<td>30s</td>
</tr>
<tr>
<td>1</td>
<td>1 min</td>
<td>72 ºc</td>
<td>1</td>
</tr>
</tbody>
</table>

RESULTS
The study included 60 patients with AOM (45% females and 55% males aged between 2-26 years.

Determination of Bacterial Frequencies by Culture and PCR
S. pneumoniae isolated from the middle ear fluid were 13%(n = 8) by culture, Out of 60 Middle ear effusion samples, lytA gene detected in (23%) 14 cases.
Out of 60 Middle ear effusion samples, cpsA gene detected in (23%) 14 cases.
The results of electrophoresis lytA and cpsA genes are shown in fig 1 and fig 2
Fig. 1. Detection of lytA gene by PCR. The sizes of PCR products are: (76 bp)
M: marker 50 bp
P: positive control
N: negative control
1,2,3: the positive samples of patients.

Fig. 2. Detection of cpsA gene by PCR. The sizes of PCR products are: (160 bp)
M: marker 100 bp
P: positive control
N: negative control
1,2,3,4,5: the positive samples of patients
Antimicrobial Susceptibility of Bacterial Isolates

The antimicrobial resistance of 8 bacterial isolated from 60 samples for this antibiotics was reported:
- vancomycin (22%),
- amoxicillin/clavunic acid (22%),
- cotrimoxazole (77.8%),
- amoxicillin (55%),
- tetracyclin (56%),
- rifampine (44.4%),
- erythromycin (67%),
- chloramphenicol (56%),
- ciprofloxacin (56%)
- and levofloxacin (0%)

Minimal inhibitory concentrations (MICs) for penicillin (56%), ceftriaxone (23%) were reported.

Resistance to 3 or more classes of drug used to treat S. pneumoniae infections is defined as multiple drug-resistant S. pneumoniae (MDRSP).

In our study rate of (MDRSP) reported 22.2%.

22% from 8 isolates of Streptococcus pneumoniae resistance to 2 antibiotics, 33% resistance 6 antibiotics, 11% resistance to 7 antibiotics and 34% resistance to 9 antibiotics were simultaneously.

DISCUSSION

The increasing number of antibiotic-resistant pneumococcal infections can lead to higher risks of treatment failure. In our study increased resistance to all antibiotics especially cotrimoxazole, erythromycin and vancomycin and This is a serious warning to physicians in the treatment of diseases caused by these bacteria.

The results of our study and other studies indicate that the use of third generation cephalosporins are useful in the treatment of this disease.

The occurrence of drug-resistant pneumococci has been associated with a variety of factors, antibiotic consumption being one of the most important. Resistance selection has mainly occurred in pneumococci colonizing or infecting children. The frequency of children as carriers, and their exposure to antibiotics, favours the selection of drug-resistant strains.

From our results, high resistance to erythromycin (67%) and cotrimoxazole (77.8%), was reported. In study of Imöhl et al. (2010) erythromycin is generally suggested as an alternative therapy for pneumococcal infection and penicillin-sensitive individuals have reported a significant decrease in macrolide non-susceptibility among children in Germany since 2005. In contrast, in study of Kohanteb and Sadeghi, in 2007 revealed an increase in the prevalence of macrolide-resistant S. pneumoniae in Iran and many parts of the world. Inappropriate administration, excessive and overdose use of erythromycin and other macrolides for treatment of pneumococcal infections may be the major factors for the elevated prevalence of macrolide resistance in our country and elsewhere.

In our study, 77.8% of the isolates showed high resistance to cotrimoxazole and 56% resistance to tetracycline. In a study by Shah et al. (2009) in Nepal the cotrimoxazole resistance was seen in 68% of isolates, which is similar to the rate in our study. This high resistance to cotrimoxazole and tetracycline is possibly due to Ease of use, Repeated administration, cost-effectiveness and Easy access over the counter. These results should serve as a warning to pediatricians in Iran, because the most common drugs used for treatment are at an extremely high in vitro resistance. Restricted antibiotic use and continued surveillance for antibiotic resistance are essential factors to solve the problem of high prevalence of antimicrobial resistance in Iran. Our isolates showed 23% resistance to ceftriaxone. In a study by Haghhighat in (2006) all S. pneumoniae isolates reported in their study were sensitive to ceftriaxone and they Reaffirmed that a third generation of cephalosporin such as ceftriaxone should be considered for empirical therapy of children with pneumococcal disease in Iran. In our study all the isolates were sensitive to levofloxacin and resistance to vancomycin wase reported 22%. Increase of vancomycin resistance in this community-acquired bacterium would be a concern. Loss of function of the VncS histidine kinase of a two-component sensor-regulator system in S. pneumoniae produced tolerance to vancomycin and other classes of antibiotic.

Surveillance studies from 2001 to 2005 by Rodriguez et al have reported VTSP (Vancomycin-Tolerant Streptococcus pneumonia) prevalences ranging from 0% to 2.6% in Sweden, Spain, Hong Kong, France.

The VTSP found in this study may possibly be attributed to any of three kinds of mechanisms: 1. defect in autolysis in one isolate, 2. the combination of TIGR4 vex2 and R6 pep27 alleles.

FUNCTIONAL DEFECT IN THE VNCS-VNCR SYSTEM.

Based on our results, 22.2% of isolates were multidrug-resistant S. pneumoniae. Resistance to 3 or more classes of Antibiotics used to treat S. pneumoniae infections is defined as multiple drug-resistant S. pneumoniae (MDRS). Based on study Lalitha et al in 2002 Multidrug resistant S. pneumoniae is increasingly being reported from many parts of the worldwide. Strains with reduced susceptibility to penicillin usually show
cross-resistance to other antibiotics and such cross-resistance was observed with tetracycline, ciprofloxacin, erythromycin, and chloramphenicol. The increasing multidrug resistance of S. pneumoniae bears important clinical implications, worldwide. Antibiotics are freely available within urban areas of Iran, and self-medication is common during a febrile illness. Based on some reports, pre-hospitalization antibiotic use affects isolation of S. pneumoniae in culture, which results in underestimation of disease burden [30].

**CONCLUSION**

S. pneumoniae is an important cause of diseases, especially in children and the elderly. The emergence of a progressive multi-drug resistance is a major concern. The data obtained in this article and related publications emphasize the hasty need to control the proper use of antibiotics to decrease the antibiotic resistance. Increase of vancomycin resistance in this community-acquired bacterium would be concern.

The experiment in diagnostic microbiology is vital for detecting man. Continued surveillance of antimicrobial resistance, serotypes and genotypes is crucial in providing information on the emergence of multiresistant clones. These data are also essential for the development of appropriate guidelines for empirical therapy of pneumococcal infections and for the inclusion of emergent serotypes in the new generation of conjugate vaccines.

**ACKNOWLEDGEMENT**

This work was partly supported by Research Center for Infectious Diseases and Tropical Medicine shahid beheshty University of Medical Sciences.

**REFERENCES**


