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ORIGINAL ARTICLE

Molecular Detection of *Helicobacter pylori* in Children with Otitis Media with Effusion at Assiut University and Sohag Teaching Hospitals

[1]Mohamed S. Abd Elaal, [2]Mohamed A. Alfeky, [1]Mohamed M. Osman, [3]Essam Abo ELmagd, [1]Tarek A. Hassan and [2]Amal A Elkhawaga*

[1]Otolaryngology Department, Faculty of Medicine, Assiut University
[2]Medical Microbiology and Immunology Department, Faculty of Medicine, Assiut University
[3]Otolaryngology Department, Faculty of Medicine, Aswan University

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Corresponding Author:
Amal A Elkhawaga
Medical Microbiology and Immunology Department, Faculty of Medicine, Assiut University
Tel.: 01065605955
Amy.elkhawaga@aun.edu.eg
Amy.elkhawaga@yahoo.com

ABSTRACT

Background *Otitis media with effusion (OME) is a commonest cause of hearing loss in childhood especially in developing countries.* ***Objectives and methodology*** *the study included 50 patients with OME. In all cases, myringotomy operation was done. It aimed to investigate the prevalence of aerobic and anaerobic bacteria in children with OME, to determine the presence of H. pylori in middle ear effusions (MEE) using polymerase chain reaction (PCR) and to examine the antibiotic susceptibility profile of isolated bacterial strains.* ***Results*** *Staphylococcus aureus (28%) was the most prevalent, followed by Peptostreptococcus species (19.2%), Coagulase negative staphylococci (17.5%), Klebsiella species (8.7%), E. coli (5.2%), and Pseudomonas aeruginosa (3.2%). H. pylori could be detected in (5.2%) using PCR. Regarding antibiotic sensitivity, most isolated strains were sensitive for ciprofloxacin and chloramphenicol while they were resistant to cephalothin except Peptostreptococcus species.* ***Conclusion*** *the obtained results indicate that H. pylori may play a role in the pathogenesis of OME.*

INTRODUCTION

Otitis media with effusion (OME) is one of the most common infections in pre-school aged children. Several hypotheses tried to explain the etiology and pathogenesis of OME. Factors related to the Eustachian tube play a major role in the development of OME, such as variation in position (more horizontal), in length (shorter), or in development (deficiency in cartilage formation) [1]. Gastroesophageal reflux disease (GERD) is considered a predisposing factor in most upper respiratory route problems including pharyngitis, rhinosinusitis, and otitis media. During childhood, the incidence rate of OME reaches up to 40% in children experiencing upper respiratory tract infections more than four times a year [2]. In general, the leading bacterial pathogens in OME are *Staphylococcus aureus, Streptococcus pneumoniae* and *non-typeable Haemophilus influenzae* , *Moraxella catarrhalis* and *Streptococcus pyogenes* [3].

Nowadays, little is known about the role of *H. pylori* in extragastric diseases; however, the role of *H. pylori* in children with OME has been increasingly discussed. *H. pylori* are Gram negative, spiral-shaped, fastidious, microaerophilic bacteria. It is closely related to chronic gastritis, gastric and duodenal ulcers and gastric cancer in human beings. The fact that *H. pylori* might be responsible for OME comes from the ability to isolate it from middle ear, tonsillar and adenoidal tissue in cases of OME[4]. Identification of the specific bacterial pathogens in MEE forms the basis for the proper selection of an accurate antibiotic therapy in patients with OME. PCR is known to have a superior ability in detecting fastidious bacterial pathogens. Since the technique depends on the amplification of highly specific gene sequence in the target bacterial nucleic acid [5].

METHODOLOGY

A hospital based descriptive study was done in Assiut University and Sohag Teaching Hospitals from March 2016 to September 2018. The study was approved by the Scientific Ethical Committee of Faculty of Medicine, Assiut University and an informed written consent was taken from all the participants in the study.

Patients and Sample Collection

A total of 57 middle ear effusions were obtained from 50 patients with OME, seven patients (14.0%) showed effusion in both ears. In all cases, myringotomy operation was done. The inclusion criteria were cases with middle ear effusion (MEE), age group from 2-14 years, both sexes, patients not receiving antibiotics for at least 2 weeks before the operation, on the other hand exclusion criteria were otitis externa and other types of otitis media, pregnancy or lactation, radiotherapy of temporal bone, concurrent use of any other treatment and presence of other chronic disease e.g. diabetes mellitus, hypertension, neoplasia, or renal problems. Each patient was examined generally and full

otolaryngological examination was done including microscopic or oto-endoscopic examination to confirm the diagnosis.

After myringotomy and under complete aseptic condition, MEE samples were taken either by syringe or cotton swab in cases of glue and dry tab, placed in an eppendorf safe-Lock tubes contained brain heart infusion broth and sent to the laboratory of Medical Microbiology and Immunology Department Faculty of Medicine, Assiut University.

Bacterial isolation:

Collected samples were divided into three parts; the first part (1ml) was stored under -20 ^{0}C for direct detection of *H. pylori* using PCR. The second part (1ml) was placed in an Eppendorf safe-lock tubes contained brain heart infusion broth and incubated at 37^{0}C for 24 hours for isolation of aerobic bacteria; the third part (1 ml) was placed in cooked meat medium and incubated for 3-7 days for isolation of anaerobic bacteria. A loopful was taken from incubated brain heart infusion broth and streaked on blood agar, Chocolate agar, Mannitol salt agar, Nutrient agar, MacConkey agar and Eosin methylene blue medium. Isolation of anaerobic bacteria was done on Brain heart infusion agar and blood agar and were incubated for 2-3 days in the anaerobic Jar using the AnaeroGen™2.5L gas generating kit (oxoid Ltd, RG24 8pw).

Standard bacteriological identification of the isolated bacteria:

Isolated bacterial strains were identified according to standard microbiological and biochemical methods [6].

Molecular identification of Urease gene for detection of *H. pylori* by PCR [5]:

DNA was extracted from samples using GeneJET Genomic DNA Purification Kit (Thermo Fisher scientific) according to the manufacturer's instructions. Detection of *H. pylori* in extracted samples, using PCR was performed with specific primers targeted urease gene regions as described in table 1. The PCR was performed in 50 µl reaction volume in an Eppendorf Thermocycler (Applied Biosystems®, USA). Reaction mixtures contained 0.4 µM (each) primer, 0.2 µM (each) deoxynucleoside triphosphate (dATP, dGTP, dTTP and dCTP) (Gibco BRL) and 1X PCR reaction buffer 1.5 µM, Mgcl2 with the addition of 10 µl of extracted DNA. DNA from *H. pylori* [7] and a tube containing water in place of DNA was assayed in each PCR run as positive and negative controls respectively of the reaction.

Ten-microliter of each PCR products were subjected to gel electrophoresis 1.5% agarose (Ultra-Pure; Gibco BRL) in Tris-acetate-EDTA buffer for one hour. The gel was stained in ethidium bromide solution (1 µg/ml) for 10 minutes. The results were analyzed according to the product size, which were visualized by UV transilluminator and photographed.

Table 1: Target genes, primer sequence and PCR protocol

***H. pylori* Urease C gene primer**	**Sequence (5'-3' direction)**
Urease C-F	TGGGACTGATGGCGTGAGGG
Urease C-R	AAGGGCGTTTTTAGATTTTT
Cycle profile (35×)	95ºC/1min→53ºC/1min→72ºC/1min→72ºC/5min
PCR product size	820

Antimicrobial susceptibility testing:

Antimicrobial susceptibility profile was carried out on Muller-Hinton agar (Oxoid, England) using Kirby–Bauer disc diffusion method, according to recommendations of National Committee for Clinical Laboratory Standards CLSI [8]. Ciprofloxacin 5 mcg, Vancomycin 30 mcg**,** Gentamicin 10 mcg**,** Chloramphenicol 30 mcg Amoxicillin 25 mcg, Cefoxitin 30 mcg, Erythromycin 15 mcg, Cephalothin 30 mcg, Ampicillin 10 mcg, Tetracycline 30 mcg, and Clindamycin 2 mcg were evaluated.

Statistical Analysis:

Statistical package for social sciences (IBM-SPSS), version 24 IBM- Chicago, USA (May 2016) was used for statistical data analysis. Data expressed as mean, standard deviation (SD), number and percentage. Mean and standard deviation were used as descriptive value for quantitative data, while number and percentage were used to describe qualitative data.

RESULTS

Table 2: Demographic data in study cases.

Item	**Descriptive "n=50"**
1- Age "yrs."	6.73±2.16
2- Sex:	
• Male	29 (58%)
• Female	21(42.0%)

From 57 MEE samples obtained in this study, the demographic data are reported in table 2

Aerobic and anaerobic bacteria isolated by conventional methods are presented in figure 1 and photo 1.

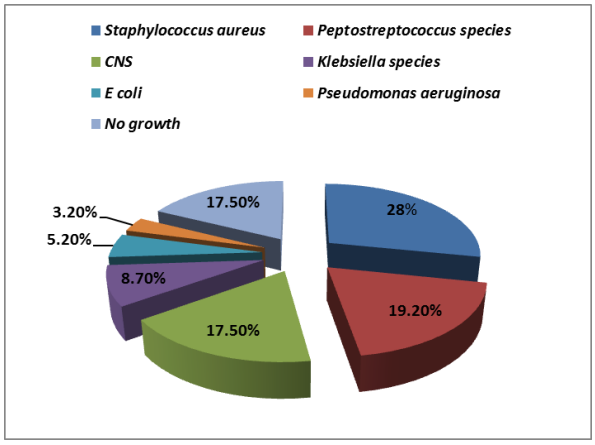

Fig 1: Prevalence of different bacterial strains isolated by conventional method

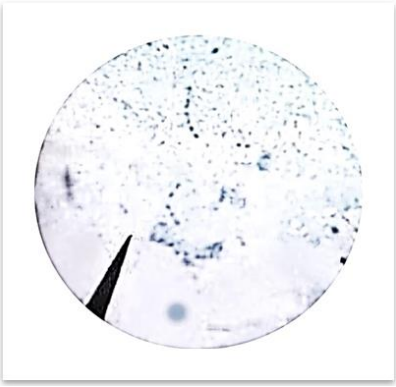

Photo 1: Film from brain heart agar stained with methylene blue showing *Peptostreptococcus species*

Anaerobic *Peptostreptococcus species* was identified conventionally by Gram stain, colony morphology and biochemical reaction, it is Gram stain positive, spherical, or ovoid; arranged in pairs, tetrads, or chains, catalase negative, urease variable, H_2S-positive, nitrate reduction occurs and indole test-negative.

H. pylori could be detected only in 3 MEE samples as described in photo 2.

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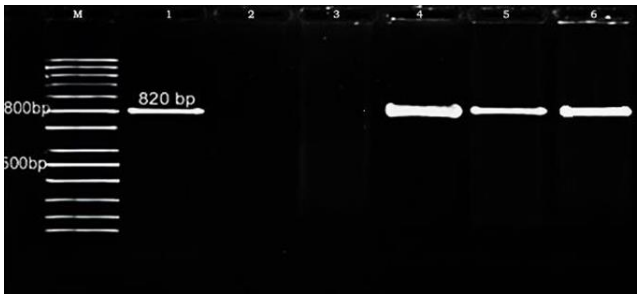

Photo 2: Results of PCR for detection of *H. pylori* in MEE samples.
M= 100 bp marker, **1**= Positive control; **2**= Negative control; **4, 5** and **6** = positive samples for *H. pylori*

Antibiotic sensitivity pattern of the different isolated bacterial strains is shown in table 3 and figure 2.

Table 3: Antibiotic sensitivity testing for the isolated bacterial strains

Item	*Staph. aureus* "n=16"	*Peptostreptococcus species* "n=11"	*CNS* "n=10"	*Klebsiella species* "n=5"	*E. coli* "n=3"	*Pseudomonas aeruginosa* "n=2"
DA						
I	10(62.5%)	7(63.6%)	1(10%)	0	0	1(50%)
R	4(25%)	4(36.4%)	4(40%)	2(40%)	0	1(50%)
S	2(12.5%)	0	5(50%)	3(60%)	3(100%)	0
TE						
I	5 (31.2%)	2(18.2%)	0	1(20%)	0	0
R	1 (6.2%)	9(81.8%)	4(40%)	0	1(33.3%)	1 (50%)
S	10 (62.5%)	0	6(60%)	4(80%)	2(66.67%)	1 (50%)
AM						
I	5 (31.2%)	8 (72.7%)	2(20%)	0	0	0
R	10 (62.5%)	1 (9.1%)	8 (80%)	5 (100%)	2 (66.7%)	2 (100%)
S	1 (6.2%)	2 (18.2%)	0	0	1 (33.3%)	0
KF						
I	0	2(18.2%)	0	0	0	0
R	16(100%)	0	10(100%)	5(100%)	3(100%)	2(100%)
S	0	9(81.8%)	0	0	0	0
E						
I	6(37.5%)	3(27.3%)	2(20%)	0	0	2(100%)
R	6(37.5%)	8(72.7%)	5(50%)	5(100%)	2(66.7%)	0
S	4(25%)	0	3(30%)	0	1(33.3%)	0
AX						
I	4 (25%)	5(45.5%)	3(30%)	0	0	1(50%)
R	10(62.5%)	1(9.%)	5(50%)	5(100%)	2(66.7%)	0
S	2(12.5%)	5(45.5%)	2(20%)	0	1(33.3%)	1(50%)
C/30						
I	5(31.2%)	2(18.2%)	2(20%)	1(20%)	0	1(50%)
R	1(6.2%)	0	0	0	0	0
S	10(62.5%)	9(81.8%)	8(80%)	4(80%)	3(100%)	1(50%)
CN						
I	7(43.8%)	8(72.7%)	0	2(40%)	1(33.3%)	0
R	5(31.2%)	2(18.2%)	5(50%)	1(20%)	0	1(50%)
S	4(25.0%)	1(9.1%)	5(50%)	2(40%)	2(66.7%)	1(50%)
VA						
I	3(18.8%)	7(63.6%)	2(20%)	0	0	1(50%)
R	4(25%)	4(36.4%)	2(20%)	5(100%)	3(100%)	0
S	9(56.2%)	0	6(60%)	0	0	1(50%)
FOX						
I	3(18.8%)	2(18.2%)	0	0	0	0
R	10(62.5%)	1(9.1%)	9(90%)	5(100%)	3(100%)	1(50%)
S	3(18.8%)	8(72.7%)	1(10%)	0	0	1(50%)
CIP						
I	2(12.5%)	5(45.5%)	0	0	1(33.3%)	0
R	7(43.8%)	1(9.1%)	3(30%)	2(40%)	0	0
S	7(43.8%)	5(45.5%)	7(70%)	3(60%)	2(66.7%)	2(100%)

CIP: ciprofloxacin, **VA**: Vancomycin , **CN**: Gentamicin , **C:** Chloramphenicol , **AX**: Amoxicillin **FOX**: Cefoxitin , **E**: Erythromycin , **KF** :Cephalothin , **AM:** Ampicillin, **TE**: Tetracycline, **DA**: Clindamycin

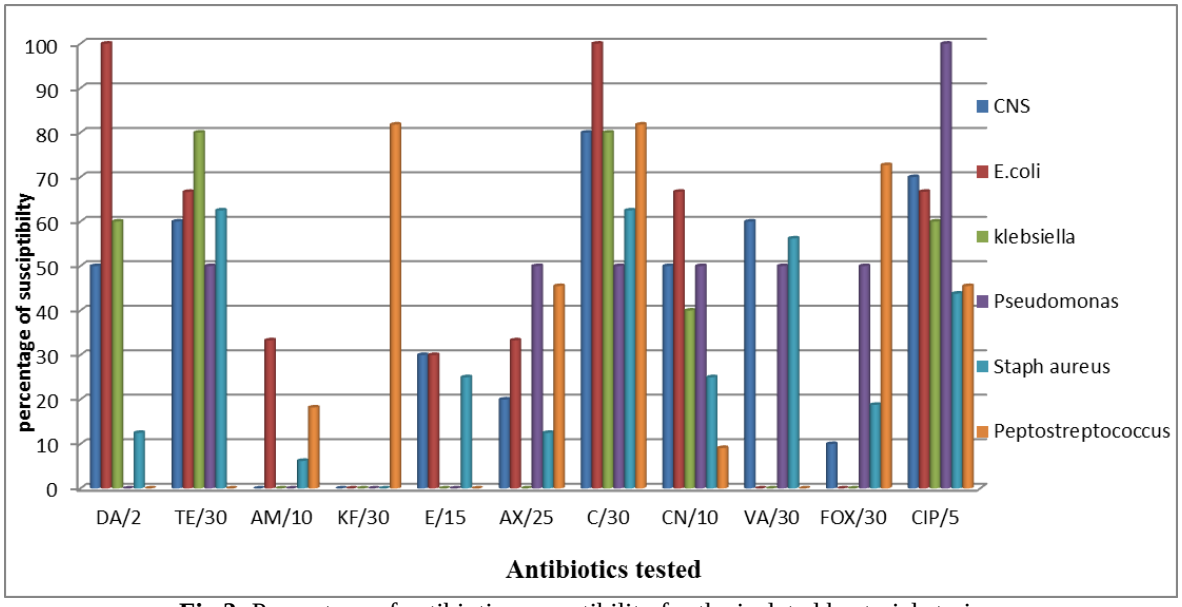

Fig 2: Percentage of antibiotic susceptibility for the isolated bacterial strains.

DISCUSSION

Although many studies have recently been done to demonstrate the etiology of OME, investigators up to date are unable to obtain a definite answer. OME is defined as the presence of a non-purulent MEE for ≥3 months with the absence of gross signs of infection. It is a prevalent disease in the pediatric population and a leading reason for conductive hearing loss in the developed world [9]. The mean age of our study group was 6.73±2.16. In contrast, another study was done on a wider variety of ages, with a mean age of 25.6 years [10]. More than half of our cases (58%) were males and 42% were females, this was consistent with a recent study that found 58.6% of their cases were males [11].

Identification of the recent causative bacterial agent in our locality is very essential for proper management of OME in children. The bacteriology of 57 samples of MEE from 50 patients was investigated. The most commonly detected organism was *Staphylococcus aureus* (28%), followed by *Peptostreptococcus species* (19.2%), then *CNS* (17.5%), *Klebsiella species* (8.7%), *E. coli* (5.2%), *Pseudomonas aeruginosa* (3.2%) finally , *H. pylori* was detected by PCR in only 3 samples (5.2%). This result differs from a recent study who found that *Pseudomonas aeruginosa* was the most predominant species (34.7%) of specimens, followed by *Staphylococcus aureus* (18.7%), *Klebsiella* aerogenes (12%), *Proteus mirabilis* (9.3%), *E. coli* (8%), Non fermenting Gram negative bacilli (5.3%) and *Klebsiella oxytoca* (1.3%) of specimens [12]. Moreover, Two Lebanese studies investigating the bacterial etiology of pediatric OME have found that *H influenzae* was the most prevalent bacteria [13]. Brook I [14] isolated *Peptostreptococcus, Fusobacterium species*, pigmented *Prevotella* and *Porphyromonas species* from chronic suppurative otitis media, further studies are needed for evaluating the role of anaerobic bacteria in OME and the proper therapeutics of these bacterial agents.

H. pylori are a fastidious microaerophilic bacteria, their growth is both difficult and time-consuming, require 3 to 7 days of incubation for isolation. Therefore, PCR is considered a rapid, sensitive, specific, low cost and useful method for detection of *H. pylori* [15]. Several studies reported the relationship between *H. pylori* and the pathogenesis of different upper aerodigestive tract diseases, especially when an induced inflammation was done in the middle ear of mice by inoculating *H. pylori* whole-cell protein lysate [16]. These findings raised the question whether *H. pylori* play a causative role in OME. To approach this issue, we investigated the presence of *H. pylori* in the MEE of our pediatric patients with OME. Herein, *H. pylori* were detected directly in MEE by PCR in only3 samples (5.26%) which is in consistent with the results obtained by Karlidag et al. [17] who found among 55 ear effusion samples 9 samples (16.3%) were positive for *H.* pylori. On the other hand Baitar et al. [18] failed to detect *H. pylori* by either culture or PCR (18). Nowadays, the association between chronic middle ear diseases and gastroesophageal reflux (GERD) has been increasingly discussed. Carr et al. reported that 42% of infants, who had undergone adenoidectomy, had GERD [19]. Also**,** Farhadi et al.[20] found *H. pylori* in adenoid tissues of 15% children with adenoid surgery (20). Stomach secretions containing *H. pylori* go up to oral cavity, then colonize tonsils, adenoid tissues and ascend to middle ear directly or through GER. Also, the pH value of middle-ear effusions in chronic OME patients was

found to be between 7.0 and 9.0. Such an alkaline environment enables *H. pylori* to survive and multiply[21].

The use of antibiotics as an empirical therapy is not recommended; identification of specific bacteria in MME forms the basis for accurate antibiotic therapy and decrease the potential risk of complications and emergence of drug resistance [22]. Isolated *Staph aureus* and CNS strains show highest sensitivity to vancomycin, ciprofloxacin and chloramphenicol while the least sensitivity was to ampicillin and clindamycin. *Pseudomonas aeruginosa* showed the highest sensitivity to ciprofloxacin while it was resistant to clindamycin, ampicillin and erythromycin. *E. coli* was sensitive for clindamycin and chloramphenicol and resistant to vancomycin and cefoxitine, *klebsiella species* was sensitive for tetracycline and ciprofloxacin and resistant to vancomycin. All isolated bacterial strains were resistant to cephalothin except *Peptostreptococcus*. Sang H. [23] reported in their study that the most frequently isolated strains were CNS followed by Methicillin resistant *Staph aureus* (MRSA) and *Pseudomonas aeruginosa.* CNS and MRSA show 100% resistance to penicillin, tetracycline and cefoxitin, on the other hand *Pseudomonas aeruginosa* show low sensitivity to quinolones .

CONCLUSION

The present study highlights the importance of both aerobic and anaerobic bacteria in the development of OME. *Staphylococcus aureus*, followed by *Peptostreptococcus species*, CNS, *Klebsiella species, E. coli*, and *Pseudomonas aeruginosa* were considered the most common causative bacterial agents of OME in our locality. Ciprofloxacin and chloramphenicol show the highest sensitivities for most isolated bacterial strains. Also, the present study pointed out the causal relationship between OME and the presence of *H. pylori* in the middle ear.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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