Effect of Combined Antibiotics and Biofilm Formation in Some Bacterial Pathogens from Otitis Media among Children in Baghdad, Iraq

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Abstract

Otitis media bacterial infections during childhood may contribute to the development of repeated nasopharyngeal infections and it is complicated of recurrent or chronic middle ear diseases, especially with multidrug resistant strains.

The results of antibiotic synergism for the most prevalent three bacterial species, isolated from otitis media infections in children, and had the highest antibiotic resistance was revealed that combinations ceftazidime-amikacin and ceftazidime- ciprofloxacin displayed synergistic activity fractional inhibitory concentration (FIC ≤ 0.5) against the most of tested isolates; *Pseudomonas aeruginosa*, *Porteus mirabilis*, and *Klebsiella pneumonia*. In the biofilm test all tested isolates were showed biofilm production by tissue culture plate method (TCP), in the present study, the strong biofilm production was most prevalent in *Pseudomonas aeruginosa* (100%) followed by *S. aureus* (71.4%). Biofilm formation increases the activity of antibiotics in children otitis media infections and using antibiotics combinations may be essential for optimum management of OM patients.

Keywords: *Otitis media, Biofilm, Antibiotics, synergistic activity.*

Introduction

Otitis media (OM) is an inflammatory disease of the middle ear, with different medical conditions and symptoms. It is caused as a result of a blockage to the Eustachian tube. In contrast with adults, the Eustachian tube is shorter and more horizontal in children and also it is consists of more flaccid cartilage, which can impair its opening, therefore otitis media is more common in children [(1)]. Recurrent Acute otitis media (RAOM) causes pain and discomfort in children, also it was noted that 20–30% of infants suffer from RAOM and approximately 70% of infants experience at least one otitis episode by the age of 2 years [(2)]. Multiple otopathogens can colonize the middle ear and nasopharyngeal. The bacterial attachment and colonization, biofilm formation, and invasion of the middle ear are enhanced by the viral infections of the nasopharynx [(3)]. The main pathogenic bacteria which contribute with the middle ear infections are *Haemophilus influenzae, Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Moraxella catarrhalis*) and considered as the main risk for OM and RAOM [(4,5)]. Several studies indicated to other species whic isolated from the middle ear fluid of children as causative agents of chronic suppurative otitis media(CSOM) such as *Pseudomonas aeruginosa, Staphylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella spp* and *Escherichia coli* [(6,7)]. Most of the bacterial pathogens can form biofilm as the main step for colonization and this biofilm provide some bacteria more effective resistance and tolerance to antibiotics according to the

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complicated structure of bacterial biofilm[8]. In Iraq, there is little attention about the study of the role of bacteria species in Otitis media occurrence and their capacity to the formation of biofilm and its relation to increasing antibiotic resistance. Therefore the aims of the study are: Isolation and identification of some species of bacteria, which infect the Iraqi children with otitis media infection by using conventional and molecular method. Investigate the antibiotic resistance of these isolates and study their ability to form the biofilm as a virulence factor. Also, conducting the antibiotics synergism to detection the lowest concentrations of the combination of antibiotics to avoid resistance of bacteria.

Materials and Method

The samples of the study: The collection of study samples has taken place at the period between November 2018 and completed at end of April 2019, it has included 138 clinical specimens as ear discharge samples, collected from inpatients and outpatients with otitis media infections that admitted in four hospitals in Baghdad**,** from both gender with age ranging from 6 months to 15 years**.**

Isolation and identification of bacteria: The bacterial which selected for this study were identified by biochemical tests and Vitek2 system, these isolates include 28 of pathogenic bacterial isolates distributed as 7 from 4 species (*Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae* and *Staphyllococcus aureus*) for Quantification of biofilm formation. From the same group of bacteria, 18 multidrug resistant isolates of three bacteria species (*Pseudomonas aeruginosa, Proteus mirabilis,* and *Klebsiella pneumoniae*) were selected for evaluation the antibiotics combination

Antibiotics Synergy Test by Using Checkerboard Method: Checkerboard is method to evaluate interactions between antibiotics with serial concentrations as synergism, additive, indifferent, or antagonism [9].

Amikacin, Ciprofloxacin and Ceftazidime, each antibiotic is prepared and MICs are calculated for each antibiotic by microdilution method by using resazurin dye as described by Elshikh *et al*. (2016)[10].

The checkerboard method uses for study Synergism between Ceftazidime – Ciprofloxacin and Ceftazidime – Amikacin. The test done for most prevalent three bacterial species with the highest antibiotic resistance, which detected in otitis media cases. The concentrations were from (1 µL -64 µL) for Ceftrazidime, (0.5 µL-256 µL) for Amikacin and Ciprofloxacin. Dilution chequerboard technique was conducted to evaluate the effect of antibiotic combinations. Fractional inhibitory concentration (FIC) indices for each agent is calculated by dividing the MIC of the antibiotics when used in combination by that of the drug alone. The FIC index is the sum of the FICs of each of two antibiotics when examined in combination[11].

A minimum FIC index of ≤0.5 indicates synergy, while, if the minimum FIC index is >0.5 and ≤ 1, the effect of the combination was classified as additive. If the minimum FIC index is >1 and ≤2, the effect of the combination was classified as indifferent, and antagonistic if >2[9].

Microtitre plate assay for biofilm quantification: Biofilm was formed on 96 well flat bottom polystyrene microtitre plates as described by Kırmusaoglu (2019)[12]. Briefly, A 10 µl of cell suspension having 0.5 O.D600 nm was inoculated in 190 µl Tryptic soy broth medium in each well. Tow hundred µl of sterile distilled water was added in peripheral wells to reduce the water loss. Then microtitre plate was incubated at 37 ºC for 18 h. After aspiration of planktonic cells, biofilms were fixed with 99% methanol. Plates are washed twice with phosphate buffer saline or sterile saline water and air dried. Then, 200 µl of crystal violet solution (0.2%) was added to all wells and the excess crystal violet was removed after 5 min, and plates were washed twice and air dried. The cell bound crystal violet was dissolved in 33% acetic acid. Biofilm formation was measured in terms of O.D 570 nm using micro plate reader. Cut off value (ODc) was calculated, which can provide categorization of isolates as biofilm producer or not.

Statistical Analysis: The Statistical Analysis System, SAS (2012) [13] program used in this study. According to Chi-square test uses for significant comparing between percentages. Least significant difference (LSD) test was used to significant compare between means (0.05 and 0.01 probability) in this study

Results and Discussion

The checkerboard method uses for studying Synergism between Ceftazidime- Ciprofloxacin and Ceftazidime – Amikacin. The test is done for most prevalent three bacterial species with the highest antibiotic resistance, which detected in otitis media infections. The concentrations are from (1 - 64 µg/ml) for Ceftrazidime,

(0.5 - 256 µg/ml) for Amikacin and Ciprofloxacin. The results of MICs of three antimicrobial agents and FICs of 2 combinations against 18 isolates of three bacteria species (*Pseudomonas aeruginosa, Proteus mirabilis,* and *Klebsiella pneumoniae*) are showed in Table (1).

Table (1). MIC of antibiotic combinations and synergy test results.

Isolates	Antibiotics combination	MIC of first antibiotic alone (µg/ml)	MIC of first antibiotic in combination (µg/ml)	MIC of second antibiotic alone (µg/ml)	MIC of second antibiotic in combination (µg/ml)	FIC	Result
A1	AK-CAZ	64	16	64	8	0.375	Synergy
A1	CIP-CAZ	4	4	64	16	1.25	Indifferent
A5	AK-CAZ	8	2	128	32	0.5	Synergy
A5	CIP-CAZ	32	8	128	32	0.5	Synergy
A27	AK-CAZ	64	16	64	8	0.375	Synergy
A27	CIP-CAZ	4	2	64	16	0.75	Additive
P1	AK-CAZ	32	4	32	8	0.375	Synergy
P1	CIP-CAZ	2	1	32	16	1.0	Additive
P20	AK-CAZ	64	16	64	16	0.5	Synergy
P20	CIP-CAZ	32	32	64	32	1.5	Indifferent
P23	AK-CAZ	32	4	32	16	0.625	Additive
P23	CIP-CAZ	64	16	32	8	0.5	Synergy
K4	AK-CAZ	64	16	16	4	0.5	Synergy
K4	CIP-CAZ	16	4	16	8	0.75	Additive
K14	AK-CAZ	128	64	64	32	1	Additive
K14	CIP-CAZ	32	8	64	8	0.375	Synergy
K15	AK-CAZ	128	32	64	16	0.5	Synergy
K15	CIP-CAZ	16	4	64	16	0.5	Synergy

FIC: Fractional Inhibitory Concentration; AK: Amikacin; CAZ: Ceftazidime; CIP: Ciprofloxacin; A= *Pseudomonas aeruginosa,* P = *Proteus mirabilis,* K= *Klebsiella pneumoniae.*

Results revealed combinations ceftazidime-amikacin and ceftazidime- ciprofloxacin displayed synergistic activity (FIC $\leq$ 0.5) against the most of tested isolates except tow isolates *P. aeruginosa* A 1 and *P. mirabilis* P20, which showed indifferent with the ceftazidime-ciprofloxacin combination.

In the current study, none of the antimicrobial combinations tested demonstrated antagonism against any of the isolates tested. Out of 18 isolates, 11 exhibited synergistic activities, while the additive activity showed in 5 isolates, as the result of *K. pneumoniae* K4 (Figure 1).

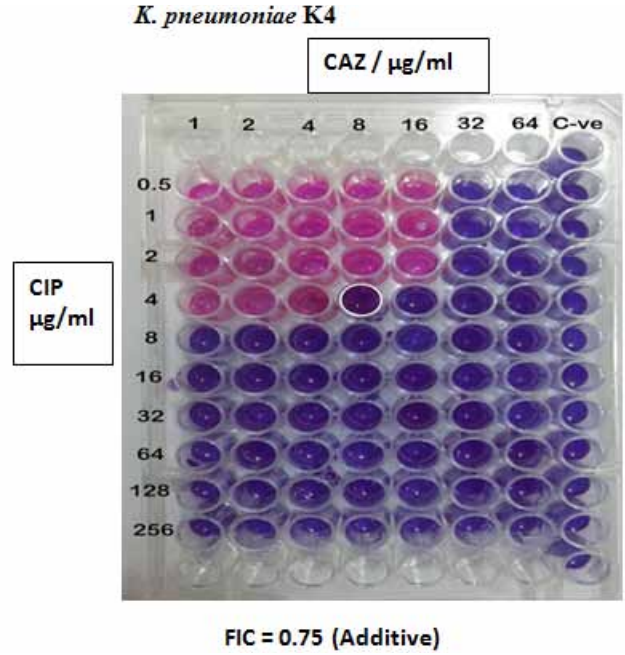

Figure (1). FIC of antibiotics combination ceftazidime-amikacin against *K. pneumoniae* K4.

The four isolates *P. aeruginosa* A1, A27, *P. mirabilis* P1, and *K. pneumoniae* K14 displayed the highest affected by the 2 combinations especially with ceftazidime-amikacin (FIC = 0.375) (Table 1).

Antibiotic combinations to the treatment the resistant infections, have been reported to be effective. One drug may overwhelm or neutralize the mechanisms of bacterial resistance, repurposing the antibiotic drug by increasing its efficacy. May be the best-known example of antibiotic synergy is the combination of clavulanic acid with β-lactam antibiotics [(14)]. Antibiotic combination therapy is used to prevent the emergence of resistant microbial strains, treat emergency cases during the process of etiological diagnosis, and to take advantage of antibiotic synergism. This is noted to be effective when two bactericidal agents are combined, but antagonism occurs *in vitro* on combination of a bacteriostatic and a bactericidal antibiotic[(15)] checkerboard technique is an *in vitro* synergy test using to determine the antimicrobial combinations activity. It is a simple to perform and remains to be a widely used technique to assess antimicrobial combinations[(16)]. Aminoglycoside and beta-lactam combinations are the most frequent used for the treatment of many infections especially due to *P. aeruginosa*. Many studies reported these synergistic combinations[(17,18)]. In this study, it is detected that the synergy was obvious between Ceftazidime and Amikacin against *P. aeruginosa* isolates. Also, many reports indicated to the combinations of quinolone and beta-lactam antibiotics and the synergism with several rates was observed [(19, 20)]. In this study, synergy was less effective in resistant strains with Ceftazidime-ciprofloxacin combination. Mayer and Nagy (1999) [(19)] revealed that the combination of third-generation cephalosporin with fluoroquinolones rarely shows synergy. In agreement with the results of current study, Hannan *et al.* (2014) [(21)] showed that among the four combinations evaluated ceftazidime-ceftazidime-meropenem, piperacillin/ tazobactam, ceftazidime-amikacin and ceftazidime-ciprofloxacin, ceftazidime-amikacin was in high rates of synergy in multidrug resistant strains of *P. aeruginosa*. Combinations of aminoglycosides and β-lactams have synergic effects against gram negative isolates of this study. The mechanism of this combination may be due to the action of β-lactams on bacterial cell wall by destruction of peptidoglycan polymers and then easily of the entry of the aminoglycosides into the bacterial cell [(22)].

Out of the total isolates of OM infections, 28 isolates with high antibiotic resistance are selected for detection the Quantitative biofilm formation by Tissue Culture Plate (TCP) method, these isolates were distributed as 7 from 4 species which include (*Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae* and *Staphyllococcus aureus*). Quantification of biofilm formation has been shown in Table (2) and Figure (2).

All isolates (100%) showed biofilm production by TCP method. Strong biofilm producers were 14 (50 %), 10 (35.7%) were moderate and 4 (14.2%) isolates were considered as weak biofilm producers. In this study, the strong biofilm production is the most prevalent in *P. aeruginosa* (7, 100%) followed by *S. aureus* (5, 71.4%), while the most of *P. mirabilis* and *K. pneumoniae* are moderate biofilm producers.

Table (2). Screening of OM pathogenic isolates for biofilm formation by Tissue Culture Plate method.

BACTERIAL SPECIES	Number of investigated isolates (%)	BIOFILM PRODUCTION				Chi-Square (χ^2)
		STRONG	MODERATE	WEAK	NON PRODUCTION	
Pseudomonas aeruginosa	7	7	0	0	0	6.34 **
Proteus mirabilis	7	1	4	2	0	2.08 NS
Klebsiella pneumoniae	7	1	5	1	0	4.52 *
Staphylococcus aureus	7	5	1	1	0	4.71 *
Total	28 (100 %)	14 (50 %)	10 (35.7 %)	4 (35.7 %)	0	6.41 **

* (P<0.05), ** (P<0.01).

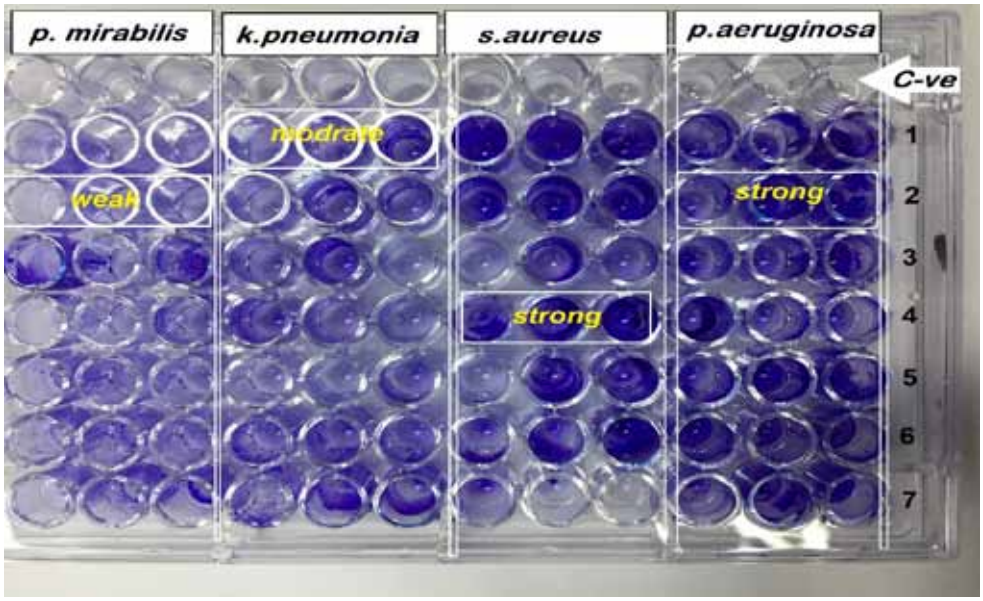

Figure (2). Detection of biofilm producers of otitis media bacterial pathogens by Tissue Culture Plate method with crystal violet staining.

In this study, the unique ability of *P. aeuginosa* to form biofim makes it as the main cause of OM infections in Iraqi children, where, Chronic suppurative otitis media is largely due to biofilm-forming bacteria, of which a common pathogen is *Pseudomonas aeruginosa*, and this species develop into biofilms and form chronic infections in the middle ear of a mouse model of Eustachian tube obstruction and acute tympanic membrane wounds[23].

The weak biofilm formation of *P. mirabilis* and *K. pneumoniae* in OM infection may be due to that these bacteria related to bacterial colonization of catheter devices infections such as urinary tract infections [24]. It finds that the mucosal biofilm has been implicated in several pediatric respiratory infections, including otitis media with effusion, tonsillitis, adenoiditis, persistent endobronchial infections, chronic rhinosinusitis, and bronchiectasis[25]. The nasopharyngeal biofilms have the main role in the effectiveness of antibiotic in children with otitis media, because the biofilm play a role in the development of chronic nasopharyngeal inflammation, which is may be associated with chronic

or recurrent middle ear disease[26]. The bacterial biofilms of clinically *H. influenzae* in patients with acute otitis media infections are responsible for the development of acute middle ear infections on the basis of the detection of biofilms produced by this species[27]. The production of biofilm may explain the failure of traditional antibiotic treatment acute otitis media, which act as favourable environment[28]. Biofilm formation is considered as a survival strategy by bacteria to antibiotics which are effective against bacteria. Biofilms are almost impossible to grow in the laboratory media and are incredibly resistant to antimicrobials, which mean that the diagnosis of chronic OM is one of the most challenging in the management of middle-ear infection[29]. The previous results gave a role of biofilm formation in the entire cavity of the middle ear of children with recurrent AOM, contributing to the viscosity of effusion[30].

Conclusion

The current study revealed that the strong biofilm was the most prevalent in *P. aeruginosa* followed by *S. aureus* and the biofilm formation leads to difficulty in treatment of OM, mainly due to its frequent association with multidrug resistant bacteria, also, it was obvious that the Combinations ceftazidime-amikacin and ceftazidime-ciprofloxacin displayed synergistic activity against the most of tested isolates.

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Ethical Clearance: Yes

Conflict of Interest: Nil

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