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RESEARCH ARTICLE

STUDY ON BACTERIAL AND MYCOTIC INFECTION OF THE MIDDLE EAR.

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Key words:-
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Abstract

Otitis media (OM) is an inflammatory disease of the mucosal lining of the middle ear and is most commonly caused by the build-up of fluid behind the ear drum, as a result of a blockage to the Eustachian tube. It is more common in the developing nations with prevalence up to 11%. The aetiology and pathogenesis of OM are multifactorial and include genetic, infections, allergy, environmental and racial factors. The Aims of the work were identification of bacteria and fungi that cause OM, determination of susceptibility of isolates to different antimicrobial agents & investigation of the possible mechanisms of antimicrobial resistance.

Patients & methods: This study was carried out in the Microbiology and Immunology Department, Faculty of Pharmacy, Zagazig University in collaboration with Ear, Nose & Throat (ENT) Department, Zagazig University Hospitals during the period from February 2013 to February 2016. 85 patients with otitis media who were attending the out-patient clinic of ENT Department were included in the study.

Results: There were 37 males and 48 females. Their ages ranged from 1.5 to 60 years. In our study *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Proteus* mirabilis, *Aspergillus niger and Aspergillus fumigatus* were the most important organisms associated with middle ear infection. For Gram negative bacteria, imipenem, amoxicillin-clavulanic acid, cefotaxime, levofloxacin and amikacin appear to be the first line antibiotics to treat chronic suppurative otitis media. For Gram positive bacteria, imipenem, vancomycin, sulfamethoxazole/trimethoprim, levofloxacin, ciprofloxacin and clindamycin appear to be the first line antibiotics in the treatment.

Conclusion: Bacteriological and mycological tests should be carried out before starting treatment of otitis media. The antibiotic susceptibility patterns must be continuously and periodically evaluated to decrease the risk of resistant strains.

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Introduction:-

Otitis media is an inflammation of the middle ear mucosa which also may involves the mastoid air cells. By the end of their first year, 86% of children will experience at least 1 episode of acute otitis media (AOM) **(Bardley-Stevenson *et al.*, 2008)**.

Global reports show that *Haemophilus influenzae* and *Streptococcus pneumoniae* are the most prevalent organisms responsible for AOM **(Sierra *et al.*, 2011)**.

Chronic otitis media (COM) is a permanent abnormality on the tympanic membrane following a long standing middle ear infection emanating from previous AOM or negative pressure to the middle ear. Chronic suppurative otitis media (CSOM) is the most severe form of OM and represents the most important cause of moderate conductive hearing loss in many developing countries **(Chirwa *et al.*, 2015)**.

A number of factors may contribute to the development of ear infections. Most experts consider Eustachian tube dysfunction to be the primary cause for both acute and chronic ear infections. Obstruction at the Eustachian tube isthmus (i.e., the narrowest portion) results in accumulation of middle ear secretions; secondary bacterial or viral infection of the effusion causes suppuration and features of AOM **(Rovers *et al.*, 2004)**.

The Aims of the work:-

This study aimed at identification of bacteria and fungi that cause otitis media, determination of susceptibility of isolates to antimicrobial agents & investigation of the possible mechanisms of antimicrobial resistance.

Materials And Methods:-

This study was carried out in the Microbiology and Immunology Department, Faculty of Pharmacy, Zagazig University on 85 patients suffering from ear discharge secondary to otitis media who were attending the out-patient clinic of ENT Department, Zagazig University Hospitals during the period from February 2013 to February 2016. All patients included in this study were not using local and/or systemic antibiotics for one week before obtaining the samples.

Ear swabs specimens were obtained, after cleaning the external auditory canal of cerumen with sterile saline and 70% alcohol swab. Specimens were handled according to the approved microbiological procedures as described by **Koneman *et al* (1997)**, transported to laboratory, examined and cultured within one hour of collection.

Ear swabs were streaked directly on a) nonselective media; Nutrient agar and Blood agar, and b) selective media; Chocolate agar, MacConkey agar, Cetrimide agar, Mannitol salt agar and Sabouraud's dextrose agar (SDA) with chloramphenicol and gentamicin then incubated at 37°C for 24 hours except of SDA plates were incubated at 30°C and the cultures paraffin sealed plates were held for 4 weeks and were examined twice weekly for growth. The isolated bacterial colonies and Candida were microscopically examined after Gram staining **(Collee *et al.*, 1996)**. Mould cultures were mounted into lactophenol cotton blue stain and examined microscopically **(Davise, 2011)**. Slide culture was prepared according to **Riddel, (1950)** for mould identification. Candida isolated colonies were presumptively identified according to their characters on SDA **(Rippon, 1982)**. Aspergillus species were cultured on Sabouraud's dextrose agar (SDA), Malt extract agar and Czapek Dox Agar. The plates were sealed by paraffin film and held for 4 weeks examined twice weekly for growth. Morphological examination of species was made with naked eye **(Johnston and Booth, 1983; Raper and Fennell, 1965)**. Further identification for isolated bacteria was done by specific biochemical reactions.

Different bacterial isolates were tested for their susceptibility to different antimicrobial agents by the Kirby- Bauer's disc diffusion method according to CLSI **(2012)** criteria. The MIC of the antibiotics carried out by broth microdilution method and interpreted according to CLSI **(2012)** guidelines.

The antifungal susceptibility tests were carried out by Etest method according to **Richard *et al* (2007)**.

According to CLSI **(2012)**, determination of methicilline resistance *Staphylococcus aureus* (MRSA), test for inducible clindamycin resistance (D-test) and phenotypic detection of β-lactamases were performed.

Effect of efflux pump inhibitor carbonyl cyanide 3-chlorophenyl hydrazone (CCCP) on susceptibility of *Pseudomonas aeruginosa* isolates to antimicrobial agents piperacillin, gentamicin, amikacin, ciprofloxacin, levofloxacin, aztreonam and cefepime was tested out according to **Abdi-Ali *et al* (2007)**.

Efflux mechanism in *Pseudomonas aeruginosa* isolates was screened by accumulation of ethidium bromide, in presence and absence of efflux inhibitor, using a modification of the methods of **Kaatz *et al* (2003)**, and **Nishino and Yamaguchi (2004)**.

Molecular identification of *Staphylococcus aureus* isolates, **a.** isolating Genomic DNA from MRSA performed according to Promega kit manufacture's instruction, **b.** PCR Assay for detection of mec-A and erm-A genes **(Birgit *et al.*, 2003)**, Electrophoresis and visualization of PCR amplicons was performed according to **Lee and Costumbrado (2012)**.

Results:-

During this study, a total of 120 isolates were obtained from 85patients. Single isolate (58.82%) were obtained from 50 specimens and double organisms (41.18%) were obtained from 35 specimens. samples were either mixed bacterial combination 27 (80%) or combination of bacteria and fungi 6 (20%).

The 94 bacterial isolates were classified to 37 (30.83%) Gram positive and 57 (47.5%) Gram negative and the 26 fungal isolates comprising 24 (20%) mould and 2 (1.67%) yeast **(figure 1)**.

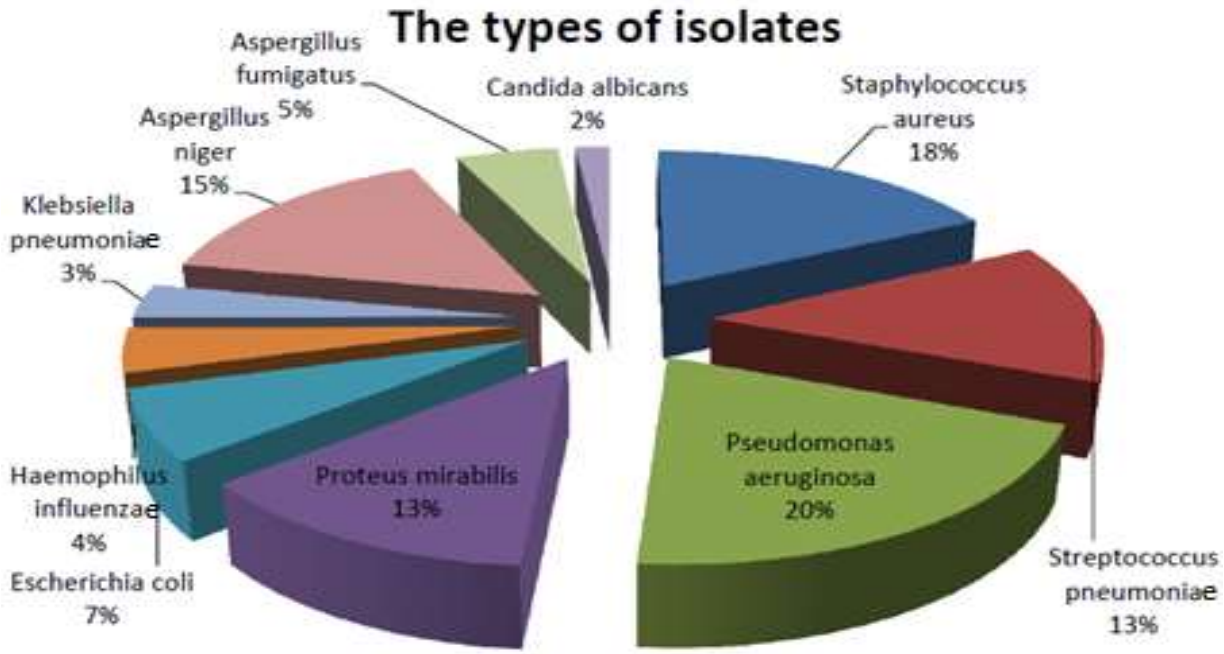

Figure (1):- The distributions of types and percentages of isolates

The bacterial isolates were tested for their susceptibility to the different antimicrobial chemotherapeutic agents by disk diffusion method; the diameters of inhibition zones were measured and interpreted according to **CLSI (2012)** guidelines **(table 1)**.

Table (1):- the susceptibility of bacterial isolates to the different antimicrobial chemotherapeutic agents

Microorganisms	E	AZM	DA	SXT	TE	IPM	AM	OX	AMC	C	CIP	CN	AK	VA	CLR	LEV	CXT	PRL	TIC	CAZ	CEF	ATM
S. aureus	12	13	17	17	4	21	0	2	16	16	16	7	11	21	-	-	-	-	-	-	-	-
Strep.pneumoniae	14	-	14	14	-	16	-	11	16	13	16	-	-	16	16	16	16	-	-	-	-	-
P. aeruginosa	-	-	-	2	4	24	-	-	20	6	5	2	3	-	-	16	8	1	12	25	21	21
P. mirabilis	-	-	-	10	4	13	-	-	14	9	10	11	13	-	-	-	15	11	-	-	15	-
E. coli	-	-	-	4	2	8	-	-	6	8	5	5	7	-	-	-	8	4	-	-	8	-
K. pneumoniae	-	-	-	0	1	4	-	-	4	4	3	1	2	-	-	4	3	2	-	-	4	-
H. influenza	-	5	-	4	-	5	1		5	4	5	-	-	-	-	5	5	-	-	-	-	5
No. of tested bacteria	46	26	47	94	73	94	26	46	94	94	94	73	73	47	16	50	73	52	25	25	52	30
Total	25	18	31	51	15	91	1	13	81	60	60	26	36	47	16	41	55	18	12	25	48	26
% of effectiveness	54.3	79	66	54.3	20.5	96.8	3.8	28.3	86.2	63.8	63.8	35.6	49.3	100	100	82	75.3	34.6	48	100	92.3	86.6

Fungal isolates were tested for their susceptibility to Itraconazole and Amphotericin B by Etest method. The filamentous fungi (*Aspergillus fumigatus* and *Aspergillus niger*) Etest results were interpreted according to **CLSI (M38-A)** while yeast was interpreted according to **CLSI (M27-A3) (table 2)**.

Table (2):- Etest ranges and results interpretation for fungal isolates to itraconazole and amphotericin B.

Microorganisms	**Itraconazole result interpretation**					**Amphotericin B result interpretation**				
	MIC range	**Sensitive**		**Resistance**		**MIC range**	**Sensitive**		**Resistance**	
		No.	**%**	**No.**	**%**		**No.**	**%**	**No.**	**%**
Aspergillus niger	**0.064 -0.25**	**18**	**100**	**0**	**0**	**0.064 -0.5**	**18**	**100**	**0**	**0**
Aspergillus fumigatus	**0.023 – 0.125**	**6**	**100**	**0**	**0**	**0.047 -0.25**	**6**	**100**	**0**	**0**
Candida albicans	**0.19 -0.25**	**2**	**100**	**0**	**0**	**0.12 - 0.25**	**2**	**100**	**0**	**0**

Test for inducible clindamycin resistance showed that, two *Staphylococcus aureus* isolates resisted to erythromycin and had intermediate resistance to clindamycin were found to be positive inducible clindamycin resistance test **(figure 2)**.

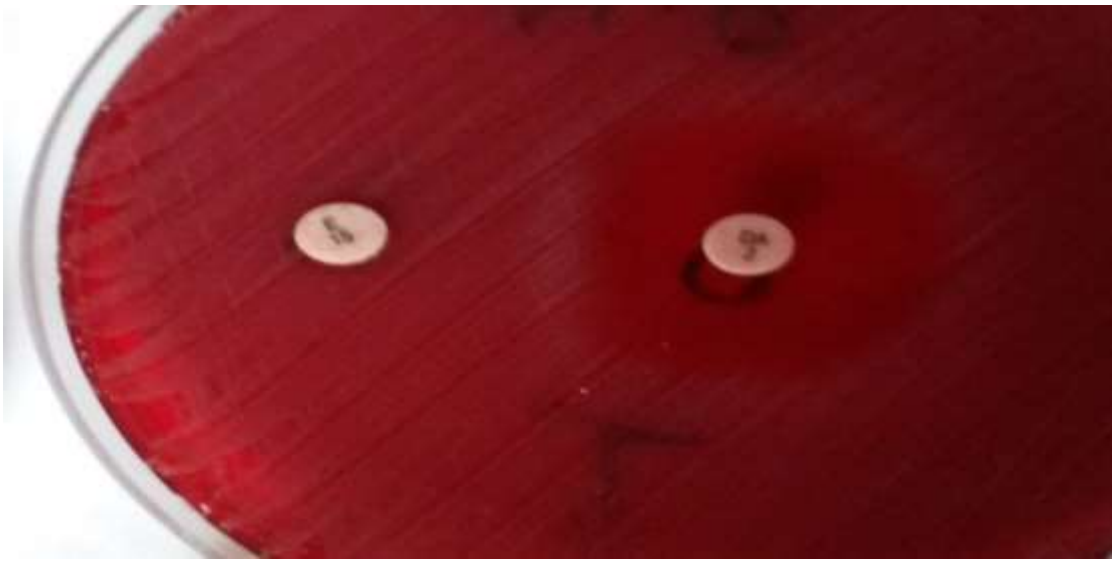

Figure (2):- D-test results for *Staphylococcus aureus*

Phenotypic detection of β-lactamases done for *Klebsiella pneumoniae* isolate and *Haemophilus influenzae* isolates. These phenotypic confirmatory tests showed one positive *Klebsiella pneumoniae* isolate that was EBLs producter and showed four *Haemophilus influenzae* isolates that were β- lactamase producer (carried out by using Cefinase disks).

Effect of efflux pump inhibitor carbonyl cyanide 3-chlorophenyl hydrazone (CCCP) on susceptibility of *Pseudomonas aeruginosa* isolates to antimicrobial agents piperacillin, gentamicin, amikacin, ciprofloxacin, levofloxacin, aztreonam and cefepime revealed that resistance of *Pseudomonas aeruginosa* isolates to these antimicrobial agents may be due to efflux activity.

The function of efflux pump were tested in 22 piperacillin resistant *Pseudomonas aeruginosa* isolates by observation of accumulation of ethidium bromide, in presence and absence of efflux inhibitor dinitrophenol. Increased fluorescence was observed in cells treated with DNP compared with control cells demonstrated the activity of efflux pump in extrusion of ethidium bromide. The toluene permealized cells was used as positive control for accumulation of ethidium bromide. All tested *Pseudomonas aeruginosa* isolates demonstrated positive efflux pump activity **(Figure 3)**.

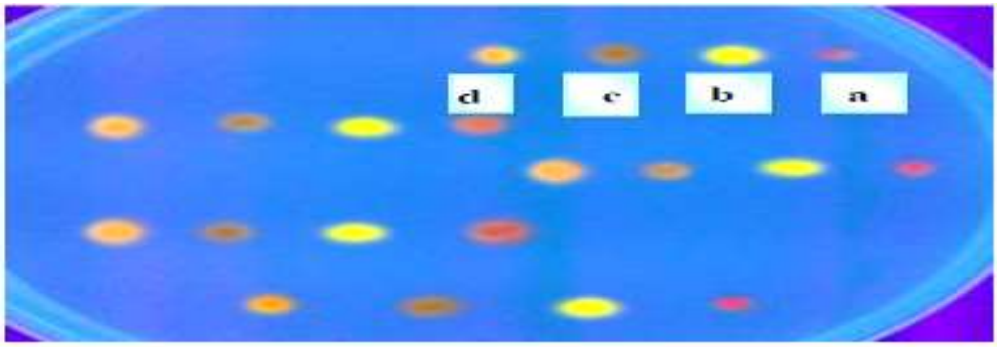

Figure (3):- Ethidium bromide accumulation in *Pseudomonas aeruginosa* cells after varies treatments; spot (a), cells treated with glucose; spot (b), cells treated with dinitrophenol; spot (c), cells without any treatment; and spot (d), cells treated with toluene.

Thirteen *Staphylococcus aureus* isolates (72.2%) were confirmed as MRSA by amplification of fragment of mec-*A* genes, each of them had band at 532bp indicating for the presence of mec-*A* gene **(figure 4)**. While 5

Staphylococcus aureus isolates (27.8%) were confirmed as MSSR as they had no bands indicating for the presence of mec-*A* gene.

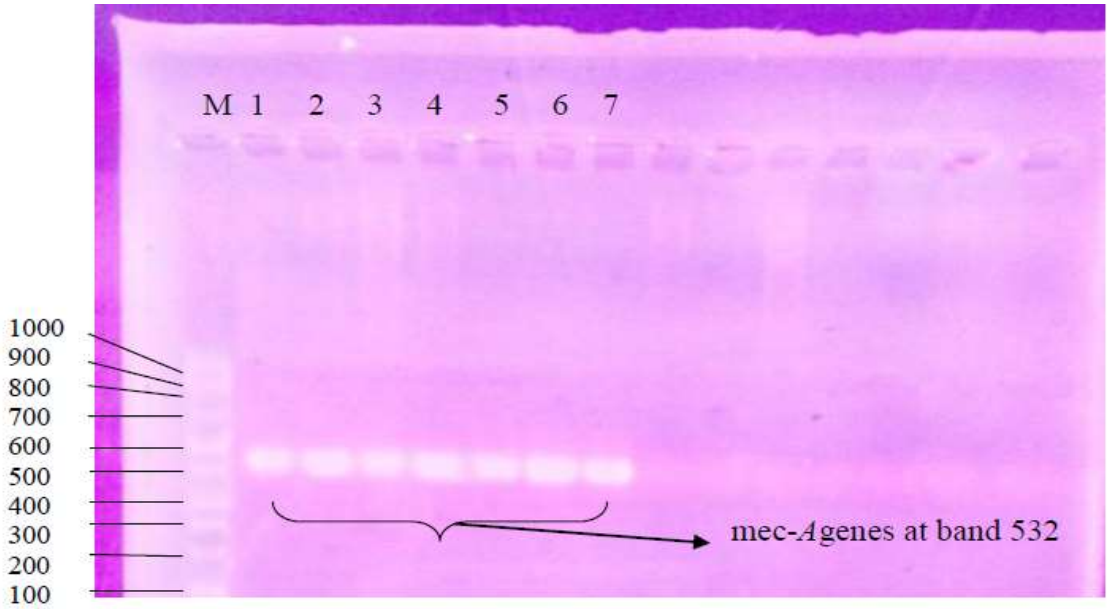

Figure (4):- Electrophoretogram of *erm*-A gene *Staphylococcus aureus* isolates

Only one *Staphylococcus aureus* isolate (12.5 %) *that* had band at 190 bp indicating for the presence of *erm*-A gene ***(figure 5)***.

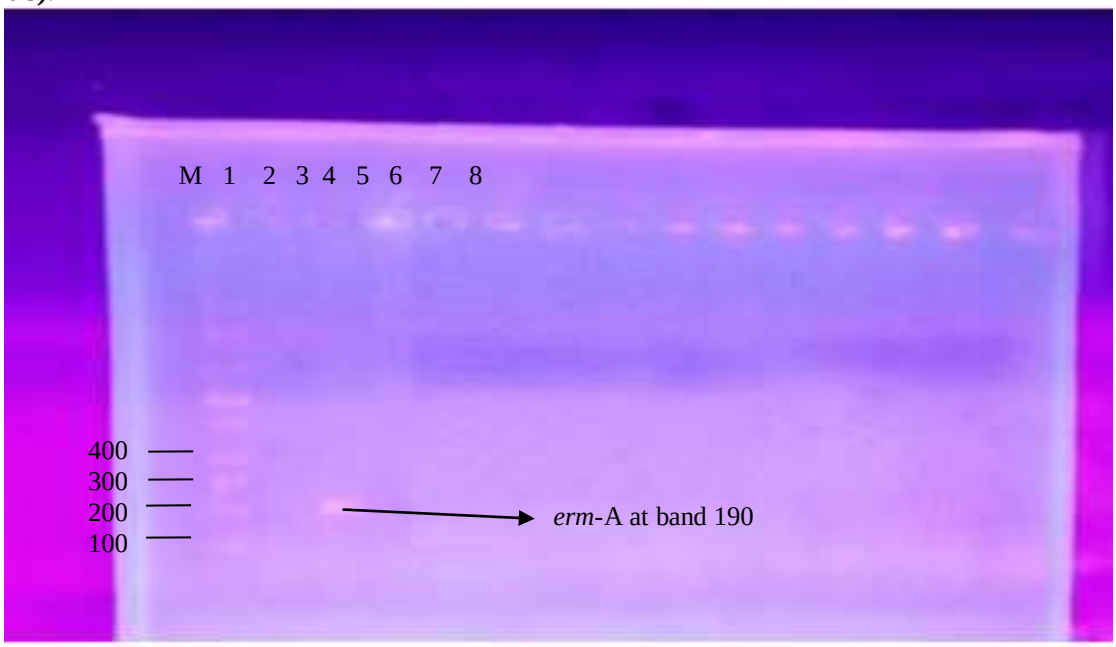

Figure (5):- Electrophoretogram of *erm*-A gene *Staphylococcus aureus* isolates.

Discussion:-

OM is a serious healthcare concern worldwide, not only because of the distress it causes to the patient and their family but also because of the substantial economic burden it imposes on the health care system **(Ologe and Nwawolo, 2002)**.

In our study, Analysis of the total 85 samples obtained from 85 patients (there diagnoses vary from AOM, COM to CSOM) revealed that mono-microbial growth was obtained in 50 (58.82 %) of total samples and 35 (41.18 %) of samples yielded poly-microbial growth, this finding agrees with the study of **Rajat *et al* (2013)** but disagree with study of **Aslam *et al* (2004)**. Difference in results could be due to different patient population & geographical variations.

In the present study, the 94 bacterial isolates (78.33% of total isolates), comprised 25 (26.6%) *Pseudomonas aeruginosa*, 21 (22.3%) *Staphylococcus aureus*, 16 (17%) *Streptococcus pneumoniae*, 15 (16%) *Proteus mirabilis*, 8 (8.5%) *Escherichia coli*, 5 (5.4%) *Haemophilus influenzae* and 4 (4.2%) *Klebsiella pneumoniae*. These findings agree with that of **Oni *et al* (2001)**. However different rates and order of isolated organisms were reported by **Okesola and Fasinam (2012)**.

In the present study, the most common causative organisms were Gram negative bacteria (47.5%) then Gram positive bacteria (30.83%). This agrees with the study of **Van Hasselt (2013)**.

In present study, the most frequently occurring organisms were *Pseudomonas aeruginosa* (20.3%) and *Staphylococcus aureus* (17.5%). This agrees with results of other authors reporting that, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were the most commonly isolated bacteria **(Mohammad *et al*., 2016; Chirwa *et al*., 2015)**. Against the present study, **Nyembue *et al* (2003)** reported that *Proteus mirabilis* and enterococci were the most frequently isolated microbes from OM.

In our study, *Proteus mirabilis* isolates represented 16% of total bacterial isolates and 12.5% of total isolates. **Maiangwa *et al* (2016)** agrees with our finding, but on the contrary **Rejitha *et al* (2015)** reported *Proteus mirabilis* in 2 isolates that represent 3.77% of total isolates.

In the present study, *Streptococcus pneumoniae* represented the second most frequently isolated Gram positive organism (13.3%). The study done by **Abdelraouf *et al* (2014)** disagrees with our study.

In the present study, *Haemophilus influenzae* isolates represented low incidence (4.2%) of total isolates. Different result reported by **Nwankwo and okeke (2014)**.

In our study, *Escherichia coli* and *Klebsiella pneumoniae* represented 6.7% and 3.3% of total isolates, respectively. Our results were comparable to that of **Chirwa *et al* (2015)**. In **Singh *et al*** study **(2012),** *E. coli* represented 4% of total infectious bacteria causes CSOM. **Nwankwo and Okeke (2014)** obtained a relatively higher rate of *E. coli* (10.2%) from otitis media samples.

In the present study, fungal isolates represented 21.67% of the total isolates. This agrees with the results of **Abd al-zaher (2004)** Different results were found in the study of **Susmita *et al* (2014)** who reported *Candida albicans* as the most common fungal isolates.

In the present study, antibiotic susceptibility tests revealed that all *Staphylococcus aureus* isolates (100%) were susceptible to vancomycin. Our finding agrees with **Abdelshafy (2015)**, showed that quinolones were the most effective antibiotic against *Staphylococcus aureus*.

In the present study, all *Pseudomonas aeruginosa* isolates (100%) sensitive to ceftazidime and 96% were sensitive to imipenem. The results agree with that of **Ogbogu *et al* (2013)**. *Pseudomonas aeruginosa* showed also high sensitivity to cefepime (84%), followed by levofloxacin (64%). Cefotaxime was less active against most *P. aeruginosa* isolates with resistance rate of 56 %. This was in agreement with the study of **Harvinder and Seth (2011)**.

In our study, Fungal isolates *Candida albicans*, *Aspergillus niger* and *Aspergillus fumigatus* were all fully susceptible to the two antifungal agents used amphotericin B and itraconazole (100% susceptibility rate). This was in agreement with the results **Abd al-zaher (2004)** stated that *Aspergillus niger* and *Aspergillus fumigatus* isolates had acceptably low MICs for amphotericin B and Itraconazole and *Candida albicans* isolates were fully susceptible to Itraconazole.

In the current study, 100% of *Staphylococcus aureus* isolates that were resistant to erythromycin and intermediated to clindamycin had inducible clindamycin resistance (positive D-test). **Yusuf *et al* (2014)** found that out 72.8% of *Staphylococcus aureus* isolated that were resistant to erythromycin and susceptible to clindamycin, were positive to D-test.

In the present study, 3.7% of *Enterobacteriaceae* isolates (*Escherichia coli, Proteus mirabilis* and *Klebsiella pneumoniae*) were found to produce extended spectrum β-lactamases (ESBLs) that found in 25% of *Klebsiella pneumoniae* isolates. These results are different from that of **Yusuf *et al* (2014)**.

In this study, Reduction in MICs (at least 2 fold) in presence of efflux inhibitor CCCP indicates the presence of proton gradient-efflux pump(s) in our *Pseudomonas aeruginosa* isolates. The highest rate for reduction in MIC was reported to aztreonam (2 to 3 fold reductions in MIC). Levofloxacin MICs decreased by 2 fold after addition of CCCP. Piperacillin, gentamicin, amikacin, ciprofloxacin and cefepime showed 2 to 3 fold reduction in MIC in presence of CCCP. These results are in agreement with that of **Adabi *et al* (2015)**.

In the current study, the piperacillin resistant *Pseudomonas aeruginosa* isolates were tested for the possible role of efflux in such resistance by testing the activity of efflux pump through demonstrating the effect of efflux inhibitor (DNP) on ethidium bromide accumulation inside cells. All 22 (100%) piperacillin resistant *Pseudomonas aeruginosa* isolates expressed positive efflux pump activity. This finding may indicate the role of efflux mechanism in resistance to piperacillin. This agrees the study of **Pournaras *et al* (2005)**.

In the present study, by PCR amplification, it was possible to demonstrate the presence of mec-*A* gene in all 18 oxacillin resistant *Staphylococcus aureus* isolates, these confirming 13 out of 18 oxacillin resistant *Staphylococcus aureus* isolates rates (72.2%) as MRSA. The high prevalence of the MRSA in the current study is in accordance with the results obtained by **Al-Khulaifi *et al* (2009)**, while **Birgit *et al* (2003)** found that all oxacillin-resistant strains carried mec-*A* gene.

In our study, by PCR amplification it was possible to demonstrate the presence of *erm*(A) gene in only one out of 4 erythromycin and/or clindamycin resistant *Staphylococcus aureus* isolates (25%). In contrast, **Birgit *et al* (2003)** found that 15 (65.2%) out of 23 strains resistant to erythromycin and/or clindamycin had the *erm*(A) gene.

Conclusion:-

In our study *Pseudomonas, Staphylococcus aureus, Streptococcus pneumoniae*, *Proteus* mirabilis and *Aspergillus species* were the most important organisms associated with middle ear infection. For Gram negative bacteria, imipenem, amoxicillin-clavulanic acid, cefotaxime levofloxacin and amikacin appear to be the first line antibiotics to treat CSOM. However, imipenem, vancomycin, sulfamethoxazole/trimethoprim, levofloxacin, ciprofloxacin and clindamycin appear to be the first line antibiotics in the treatment of Gram positive bacteria.

The antibiotic susceptibility patterns must be continuously and periodically evaluated to decrease the risk of resistant strains.

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