Study On Antimicrobial Effects of Cerumen, Human Serum, and Nanoparticles on Microbial Pathogens Isolated from Ear Infections

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ABSTRACT

Background: Bacterial infections of ear canal are widely prevalent. **Objective:** This study was planned to, diagnose cases of otitis externa then make isolation and identification of the most prevalent bacterial pathogens causing this infection. Also, to detect the phenotypic antimicrobial susceptibility testing to select the effective antimicrobials. **Subjects and methods:** A prospective clinical study was conducted on 100 cases with acute otitis externa. Patients' socio-demographic features and clinical history were recorded. Identification of the bacterial pathogens and surveying of antimicrobial resistance and resistance genes were conducted. The efficacy and minimum inhibitory concentrations (MIC) of cerumen, human serum, and silver nanoparticles against the pathogens were detected. **Results:** A total of 100 acute otitis externa patients were investigated, 25% had tonsillectomy. Most of the cases showed discomfort, itching, otalgia, and edema of the ear canal. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* were highly prevalent with rates of 30%, 20%, and 15% respectively. They were highly resistant with multiple antimicrobial resistance indices of 0.75, 0.7, and 0.95 respectively. *S.aureus* contained high rates of *mec*A1, A2, *mec*A1, *mec*C, and *BlaZ* gene. Most of *E. coli* harbored class 1 integron, *CTX*-M, and *CTX*-M-1. Most of the macrolide-lincosamide–streptogramin B methylases were present in *S.aureus* and *E.coli*. *P.aeruginosa* contained class 1 integron, while low rates of *CTX*-M, *msr*E, *mph*E. Silver nanoparticles MIC was 650 µg/mL against *E.coli* and *P.aeruginosa* with inhibition zones of 30 mm and 20 mm, respectively. The cerumen MIC was 1200 µg/mL against *S.aureus* and *E.coli* with inhibition zones of 7 mm and 9 mm respectively. Human serum was effective against *E.coli* with a MIC of 200 µL/mL and the inhibition zone was 13 mm. **Conclusions:** Bacterial pathogens of otitis externa exhibited high antimicrobial resistance rates. Cerumen, human serum, and silver nanoparticles are promising in controlling these pathogens.
Keywords: Otitis externa, Bacterial pathogens, Antimicrobial resistance, Antimicrobial alternatives.

INTRODUCTION

The ear infection, in whatever form, has diverse microbial causes that determine the effective antimicrobial medication to be selected. Infections of the external ear and the middle ear are called otitis externa and otitis media, respectively, and are considered two serious types of ear infections. Bacterial infection of the ear canal causes otitis, which occurs after a rupture in the intact skin or the protective cerumen layer in the presence of high humidity and temperature [1, 2]. It is generally referred to as the swimmer's ear. However, conditions that cause the breaking of this protective lipid barrier can allow germs to enter and multiply. Cleaning the ears with cotton buds or fingernails is considered the most common local predisposing factor has been identified as trauma [3, 4].

The Gram-positive bacteria constitute 90% of the external auditory canal and live as commensals. When protective barriers such as cerumen, diabetes, canal pH, and other immune weakened states fail to protect the ear canal, the natural flora may become pathogenic [5]. Gender, low socioeconomic level, overcrowding, ignorance about the condition, living in a slum area, poor cleanliness, bathing in pond/river water, smoking, and foreign bodies in the ear are all conditions that could predispose to suppurative ear infections [6].

Ear infections can be caused by viruses, bacteria, or fungi. *S. aureus*, *P. aeruginosa*, *Strep. pneumoniae*, *Strep. pyogenes*, *Proteus* species, and *H. influenzae* on the other hand, are mostly responsible for infection [7]. The problem of antimicrobial resistance is complex and its etiology is determined by the individual, bacterial strains, and resistance mechanisms. The evolution of resistance to newly produced antibiotics reinforces the need for new antimicrobials and lowering the uncontrolled antimicrobial utilization [8]. Because the antimicrobial resistance is growing, particularly in resource-limited nations, current research on the antimicrobial susceptibility of isolates from ear discharge is critical for effective patient management [7].

The resistance in bacteria is caused by the existence of antimicrobial resistance genes (ARGs). Pathogenic bacteria acquire ARGs via plasmid exchange and develop antimicrobial resistance. Bacterial ARG-carrying plasmids, integrons, and transposons can undergo horizontal gene transfer among similar and different bacterial strains. The death of the resistant bacteria exposes its DNA to the resistance genes, which can stay for a long period in the environment thanks to deoxynucleotide enzymes and being taken by other bacterial agents [9].

Cerumen is a hydrophobic ear wax that protects the external auditory canal epithelial lining from mechanical and microbiological attack [10]. It is a natural secretion in the external auditory canal's outer section secreted by "apocrine sweat" and the sebaceous glands

[11]. The secretion of the ceruminous glands has antibacterial and antifungal properties and is important for cleaning and lubrication of the EAC. As dead skin cells peel off and exit the ear canal, they mingle with sebaceous gland oil and ceruminous gland sweat. Cerumen is a mixture of various components, predominantly dead keratin cells. Cerumen acts as a barrier for protection and foreign particles trapping. Overall, wax is sticky, waterproof, and protective, and there should be a thin coating of wax around the canal's external opening [12].

The migration of the epithelial lining of the auditory canal takes cerumen outside, which is helped by the movement of the jaw [13]. Cerumen comprises cholesterol esters (9.6%), squalene (6.4%), wax esters (9.3%), fatty acids (22.7%), triacylglycerols (3%), cholesterol (20.9%), cholesterol sulphate (2%), ceramides (18.6%), and various unidentified polar components (7.5%). It may also contain neurostearic acid, amino acids, cerotic acid, hexone bases, triglyceride, glycopeptide, immunoglobins, copper, and other substances [14, 15]. So based on this cerumen exerts its antibacterial activity by the lysozyme content and the acidity. Blood serum's antimicrobial action is typically attributed to complement system proteins, immunoglobulins, and antimicrobial peptides. However, as the most prevalent plasma protein, albumin may interact with microbes as well [16, 17].

Silver nanoparticles have wide antimicrobial mechanisms on various bacterial pathogens. *Acinetobacter baumannii*, *Enterococcus faecalis*, and *Proteus mirabilis* make serious alterations in the cell wall and cytoplasm. Moreover, silver nanoparticles make detrimental changes on *S. aureus*, *M. luteus*, *E. coli*, *K. pneumonia*, *P. aeruginosa*, and *V. cholera* and alter membrane permeability and affects respiration in a bad manner. While, results in diatachment of the cell wall from the cytoplasmic membrane and causes plasmolysis in *L. monocytogenes*. In both *S. epidermidis* and *Salmonella typhi,* it inhibits the replication of bacterial DNA, causes damage to bacterial cytoplasmic membrane, and modifies the levels of intracellular ATP [8].

This work was planned to, diagnose cases of otitis externa then make isolation and identification of the most prevalent bacterial pathogens causing this infection. Also, to detect the phenotypic antimicrobial susceptibility testing to select the effective antimicrobials and to investigate the antimicrobial resistance genes causing the bacterial resistance. In addition to evaluate the antibacterial effects of cerumen, human serum, and silver nanoparticles as antimicrobial alternatives on each bacterial pathogen from infected cases and compare their activities with the conventional antimicrobials. That's because the new agents have no resistance genes in the bacterial pathogens.

SUBJECTS AND METHODS

This was an a prospective observational study of 100 cases of acute otitis externa that were diagnosed and treated at the ENT Outpatient Department, Benha University Hospital during the period from February 2023 to July 2023.

Calculation of sample size was performed based on the recently published report from Egypt, the total isolation rate of Gram-positive and negative bacteria 19/27 (70%) from otitis externa cases, we used the sample size calculator available at https://www.calculator.net/sample-size-calculator.html?type=1&cl=95&ci=5&pp=80&ps=113&x=62&y=15 to calculate the lower number of samples required to meet the needed statistical constraints. Hence, a total of 77 or more measurements were required to have a confidence level of 95% that the real value was within ± 5% of the measured value, so we used 100 cases for the whole study.

The inclusion and exclusion criteria with subjective, objective assessment scores, and cumulative assessment scores were performed according to **Ghanpur *et al.* [5]**.

Inclusion criteria: Patients with sudden onset of ear discomfort within 48 hours at the ENT department outpatient clinic were carefully reviewed, and the clinically confirmed patients with unilateral acute otitis externa were included in the study.

Exclusion criteria: Cases with bilateral chronic otitis externa, as well as the presence of pathology in the middle or inner ear. Individuals with congenital malformations in the opposite ear, individuals on antibiotics for ear complaints.

Severity was determined using study-specific rating systems based on symptoms and objective otomicroscopic findings. In this study, we utilized a "4-point scoring chart" to calculate subjective and objective assessment scores.

Subjective Assessment Score (SAS): It was estimated by recording scores from 0 to 2 for signs as pain (no pain recorded 0, pain on touch recorded 1, continuous pain recorded 2), itching (no itch listed 0, occasional itching during the day listed 1, itching throughout the day listed 2), discharge (no discharge assigned 0, occasional discharge assigned 1, continuous discharge assigned 2) and difficulty of hearing (non-difficult hearing was given 0, mild hearing difficulty given 1 and moderate to severe hearing loss affecting daily activities given 2). The SAS range was from 0-8.

The objective assessment score (OAS): It was calculated after the otomicroscopic findings as edema (no canal obliteration recorded 0, subtotal canal obliteration recorded 1, and total canal obliteration recorded 2), canal erythema (absent given 0 and present given 1), debris (absent listed 0 and present listed 1), and discharge (no discharge recorded 0, serous discharge listed 1, purulent discharge listed 2).

The cumulative assessment score (CAS = SAS+OAS) was recorded at the diagnosis time (CASpre) and after 48 h of topical therapy with the Ichthammol Glycerine (IG) pack (CAS48). Systemic therapy (empirical oral Amoxicillin - Clavulanate together with topical ciprofloxacin drops) was initiated in patients who did not get much relief from the IG pack (with a decrease in CAS of less than 4), and a cumulative assessment score after 7 days (CAS7) was calculated [18].

Sampling and isolation: A number of 100 patients diagnosed as otitis externa in the Outpatient Clinics, Benha University Hospital were enrolled in this study after taking their consents. The specimens were collected from the ear canal of the otitis patients using a sterile cotton swab and placed in sterile peptone water. The samples were labeled bearing the person code, date, sex, and age, and then they were transferred to the laboratory of the Microbiology Department, Benha University Hospital. For the isolation of bacterial pathogens, swabs were cultured on Cetrimide, MacConkey agar, and Mannitol salt agar plates. All media were aerobically incubated at 37ºC for 48 h.

Microbial identification and antimicrobial susceptibility testing:

The isolated bacterial agents were identified using Gram stain reaction, shape of colonies, and biochemical reactions [19]. Antibiotic susceptibility test was performed by the disc diffusion method with these antimicrobials; Amikacin (AK30), Amoxicillin and clavulanic acid (AMC30), Ampicillin (AM10), Cefixime (CFM 5), Cefoperazone (CFP 75), Cefotaxime (CTX30), Ceftriaxone (CRO 30), Cefuroxime (CXM 30), Chloramphenicol (C30), Ciprofloxacin (CIP5), Doxycycline (DO30), Erythromycin (E15), Gentamycin (CN10), Imipenem (IPM 10), Levofloxacin (LEV 5), Linezolid (LZD 30), Penicillin G (P10), Streptomycin (S10), Sulfamethoxazole/trimethoprim (SXT25), and Vancomycin (VA10). Based on the Clinical and Laboratory Standards Institute (CLSI) guidelines, the results were interpreted [20].

Detection of antimicrobial resistance genes: The bacterial isolates were refreshed onto nutrient agar media plates for 24 hrs at 37°C. The QIAamp Kit (Qiagen, Germantown Road, Germantown, USA) was utilized for DNA extraction according to the manufacturer's instructions. The used primers were *erm*(B), *erm*(C), *erm*(F), *erm*(G), *erm*(Q), *erm*(42), *mph*(E), *msr*(E), *Intl*1, *intl*2, *bla*CTX-M, and *bla*CTX-M-1. The primer sequences, amplicon sizes, and conditions of amplification were similar to **Elsayed et al.** [21].

A total volume of 25 µl was used for PCR and included 2 µl of bacterial genomic DNA (100.000 ng/µl), 12.5 µl of ready-to-use master mix supplied by (Takara Holdings, Japan), 0.5 µl of each forward and reverse primer (50 pmol/ µl) listed in Table (2) and

supplied by (Takara Holdings, Kyoto, Japan), and completed with 9.5 µl of RNase-free water.

The PCR amplification efficiency was detected by using internal positive controls from the tested *S. aureus*, *E. coli*, and *P. aeruginosa* strains, while RNase-free water was used as a negative control in each PCR experiment. The thermocycling program was modified based on the results in Table (2). To determine amplicon sizes, PCR products (5 µl) were electrophoresed on a 1.2% agarose gel, stained with ethidium bromide, and visualized with a UV transilluminator.

Cerumen collection: The cerumen was obtained from healthy and normal cases of all ages with sterile ear plugs, these cases were attended at the Hospital's ENT Outpatient Clinic after giving their consent. The cerumen suspension was 3500 g/mL (weight/volume) after being emulsified in a solution containing 30% glycerol and 5% sodium bicarbonate. The cerumen and buffer combination was then emulsified by pumping it back and forth using a sterile syringe. The cerumen suspension was stored at -20°C until microbiological testing was performed [22].

Serum collection:

A 10 mL amount of blood was collected aseptically from a peripheral vein using suitable gauge needles and a vacutainer serum separator tube. The tube was left at room temperature for at least 30 minutes to clot; trauma-induced hemolysis was avoided. To completely separate serum from blood cells, the tube was centrifuged before storage. Following centrifugation, the serum was filtered using a syringe filter and placed under complete aseptic conditions into plastic freezer vials with leak-proof screw caps.

Biosynthesis of silver nanoparticles (AgNPs):

Penicillium digitatum isolate was employed in the production of silver nanoparticles by the reduction of silver nitrate. After overnight incubation, the culture filtrate with silver nitrate solution changed color to extreme brown, whilst the control showed no color change [23]. The shape and size of the generated silver nanoparticles were detected by transmission electron microscopy micrographs acquired using prepared grids. The micrographs revealed that the particles were spherical in form and range in size from 18 to 32 nm. A similar results showed that the particles of silver nanoparticles were spherical and polydisperse [24].

Microbiological testing of collected cerumen samples Agar well diffusion technique: A volume of 1 mL containing 1×10^6 CFUs was spread onto the entire surface of the Muller-Hinton agar plate using a sterile cotton swab. Then, a sterile cork borer was used to make a hole with a diameter of 6 to 8 mm, and a volume (100 µL) of the antimicrobial agent at the minimum inhibitory concentration (MIC) was poured into the wells. Then, agar plates were incubated under aseptic conditions at 37 ºC for 24 h. The inhibition zone was

measured using a ruler and recorded for each antimicrobial [25].

Detection of the minimum inhibitory concentration:
It was implemented according to the guidelines of CLSI, and modified to the described technique of Stark, as follows: A number of 30 (20 mL falcon) tubes containing Muller Hinton Broth medium, 10 tubes contained concentrations ranged from 700-1500 µg/mL of ear wax, the second 10 tubes contained 300-750 µg/mL of silver nanoparticles, and the third 10 tubes contained 100-100 µl/mL were suited for each product. All the bacterial isolates under test were refreshed onto Muller Hinton agar plates and incubated at 37°C for 24 h. From each plate, a few colonies were transferred (using sterile cotton swabs) to 50 mL falcon tubes containing Muller Hinton broth and incubated at 37°C for 24hr. The bacterial concentration was adjusted to 1×10^6 colony-forming units (CFUs) per 1 mL by a spectrophotometer. After that, a volume of 1 mL containing 1×10^6 CFUs of various bacterial isolates was transferred to 1.5 mL Eppendorf tubes, followed by subsequent centrifugation and decanting of the supernatant (in a container containing 70% ethyl alcohol), then the tubes contained bacterial pellets. A number of 10 Eppendorf tubes were prepared for each isolate and contained various 10 concentrations of the tested substances and incubated at 37 °C for 24 h. The tubes were subjected to further centrifugation followed by decanting the supernatant media with different chemical compounds, and new Muller Hinton Broth free from chemicals was added to every tube, then the tubes were incubated for 24 h at 37 °C. The tubes were visualized for the existence or absence of turbidity, which existence elucidates the viability of bacterial cells. The minimum inhibitory concentration (MIC) was detected as the lowest concentration that stopped the bacterial growth without any turbidity. Finally, the results were recorded for each bacterial isolate with the cerumen, human serum, and silver nanoparticles [20, 26].

Ethical approval: The patients signed written informed consents to take part in the study. The research was conducted in line with the 1975 Helsinki declaration and its regulations. The Institutional Ethical Committee approval was gained under the number RC.7.12.2022 and the study was started. The study was conducted in accordance with Declaration of Helsinki.
Statistical Analysis
The SPSS statistics software for windows version 17.0 (Chicago) was implemented to detect recovery of the rates of Socio-demographic features and clinical history of patients with clinical otitis externa, isolation rates of bacterial pathogens, sensitivities to various antimicrobials, multiple antibiotic resistance (MAR) indices, and frequencies of antimicrobial resistance gen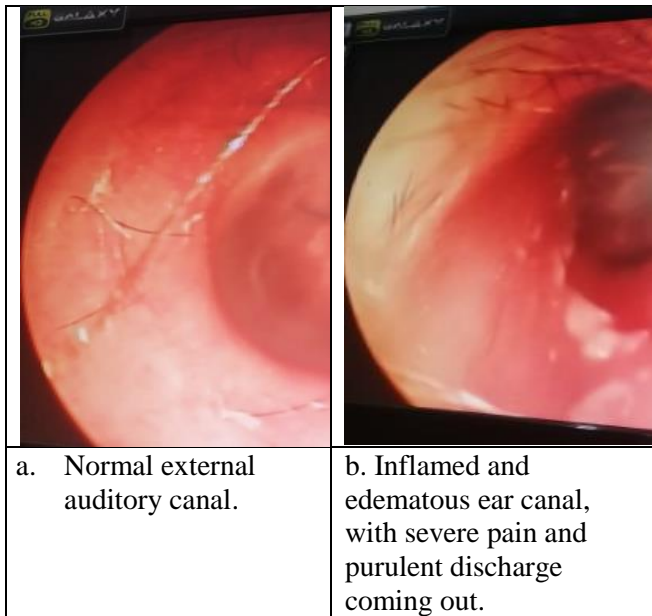es. It was also used to detect significance of differences between the gained rates (P ≤ 0.05 was considered significant).

RESULTS
100 patients from different localities of Qalyubia Governorate suffering from clinical otitis externa, 50 males and 50 females were implemented in this study figure (1). The age range of tested cases was 30-65 years, from them 90% were married and 10% were single ($p < 0.05$). Most of the patients were non-employed with a rate of 60% higher than the employed cases at 40% and the same scenario was found with smoking history as most of the cases were smokers 58% when compared with the non-smoking 42% ($p < 0.05$). Most of patients expressed no previous surgery with a rate of 72%, which was higher than tonsillectomy (25%), a denotonsillectomy 2%, and drug allergy 1% ($p < 0.05$). Edema of the ear canal was reported in all patients (100%), otalgia in 98%, discomfort in 97%, and itching in 90% ($p < 0.05$) (Table 1).

Table (1): Socio-demographic features and clinical history of patients with clinical otitis externa

Aspects of difference		Number	Percentage
Gender	Male (30-65 years)	50	50%
	Female (30-65 years)	50	50%
Marital status	Single	10	10%
	Married	90	90%
Occupation	Employed	40	40%
	Non employed	60	60%
Smoking history	Smoking	58	58%
	Nonsmoking	42	42%
Previous medical history	Adeno-tonsillectomy	2	2%
	Drug allergy	1	1%
	Tonsillectomy	25	25%
	No previous surgery	72	72%
Signs and clinical manifestations	Discomfort	97	97%
	Itching	90	90%
	Otalgia	98	98%
	Edema of ear canal	100	100%

a. Normal external auditory canal.	b. Inflamed and edematous ear canal, with severe pain and purulent discharge coming out.

Figure (1): Otoscopic views of the normal auditory canal and the most commonly found changes in it in otitis externa patients**.**

From the results of isolation and identification, table (2) showed that *P. aeruginosa* expressed the highest prevalence among the examined patients with a rate of 30%, followed by *S. aureus* with a percentage of 20%, and finally, *E. coli* represented 15% of the examined patients. There was a significant difference among them ($p < 0.05$). While, the other 35% of tested patients were negative for bacterial isolation suggesting other causative agents.

Table (2): Results of isolation and identification of various bacterial agents from otitis externa patients

Bacterial agents	Signs (Otitis externa)		%	Locality
	Male	Female		
S. aureus	10	10	20%	Qalyubia Governorate
E. coli	7	8	15%	
P. aeruginosa	15	15	30%	
Non-bacterial pathogens	18	17	35%	

Antimicrobial susceptibility and multiple antimicrobial resistance index: The isolates of *S. aureus* were sensitive to 6 antimicrobials and were resistant to 14 types. The effective antimicrobials were amoxicillin and clavulanic acid, cefoperazone, ciprofloxacin, gentamycin, vancomycin, and imipenem with rates of 8 (40%), 18 (90%), 12 (60%), 17 (85%), and 4 (20%) respectively. *S. aureus* expressed the lowest resistance and the lowest MAR index, which represented 0.7. The *E. coli* isolates showed the highest resistance, they were only sensitive to imipenem with a rate of 14 (93.3%) and resistant to 19 antimicrobials with a MAR index of 0.95. *P. aeruginosa* isolates expressed resistance to 5 antimicrobials that were ciprofloxacin, imipenem, levofloxacin, penicillin G. and sulfamethoxazole/trimethoprim with rates of 27 (90%), 28 (93.3%), 28 (93.3%), 3 (1%), and 8 (26.7%) respectively. They were resistant to 15 antimicrobials with a MAR index of 0.75 (Table 3). There was a significant difference between sensitivities and MAR indices with ($p < 0.05$).

Table (3): Results of antimicrobial susceptibility testing

Organism	AK 30	AMC 30	AM 10	CFM 5	CFP 75	CTX 30	CRO 30	CXM 30	C 30	CIP5	DO3 0	E 15	CN 10	IPM 10	LEV 5	LZD 30	P 10	S 10	SXT 25	VA 10	MAR index
Staphylo-coccus aureus	R	**S 8 (40%)**	R	R	S 18 (90%)	R	R	R	R	S 18 (90%)	R	R	S 12 (60%)	S 17 (85%)	R	R	R	R	R	S 4 (20%)	0.7
E. coli	R	R	R	R	R	R	R	R	R	R	R	R	R	S 14 (93.3%)	R	R	R	R	R	R	0.95
Pseudo-monas aeruginosa	R	R	R	R	R	R	R	R	R	S 27 (90%)	R	R	R	S 28 (93.3%)	S 28 (93.3%)	R	S 3 (1%)	R	S 8 (26.7%)	R	0.75

S: sensitive;   R: resistant

Antimicrobial resistance genes:

The methicillin-resistance genes were highly prevalent in *S. aureus*, *mec*A1, A2, *mec* A1, and *mec*C were present with rates of 14/20 (70%), 14/20 (70%), and 13/20 (65%) respectively. This result confirmed that most of *S. aureus* isolates were methicillin-resistant (MERSA). The *Bla*Z gene responsible for penicillin resistance was present with a rate of 10/20 (50%).

The macrolide-lincosamide–streptogramin B methylases *erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(G), and *erm*(Q) were present with rates of 13/20 (65%), 3/20 (15%), 20/20 (100%), 1/20 (5%), 0/20 (0.0%), and 13/20 (65%) respectively. There found a complete correlation between the existence of these genes and the complete resistance to erythromycin. A significant difference among the non-similar rates was present ($p < 0.05$).

Based on antimicrobial resistance genes in *E. coli* isolates, the class 1 integron was highly prevalent among isolates (93.3%) compared to class 2, which was not found at all. The cefotaxamases *CTX*-M and *CTX*-M-1 were highly prevalent among isolates with rates of 80% and 53.3% respectively. The macrolide-lincosamide–streptogramin-B methylases distribution patterns were 100%, 40%, 86.7%, 46.7%, 86.7%, and 80% for *erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(G), and *erm*(Q) respectively. There was a complete correlation between the existence of these genes and the complete resistance to erythromycin. A significant difference among the non-similar rates was found ($p < 0.05$).

For *P. aeruginosa*, the class 1 integron was found in all isolates, while class 2 was absent. The *CTX*-M was present in 6/30 (20%) and *CTX*-M-1 was absent. The macrolide resistance genes, *msr*E and *mph*E were present in 1/30 (3.3%) and 3/30 (10%) of isolates, respectively. A weak correlation between the existence of these genes and the complete resistance to erythromycin was present. A significant difference existed among the non-similar rates ($p < 0.05$).

The three antimicrobial alternatives exhibited efficacy on otitis externa bacterial pathogens. Silver nanoparticles was effective with MIC of 650 µg/mL against *E. coli* and *P. aeruginosa* and the inhibition zones were 30 mm and 20 mm respectively. The ear wax (cerumen) was effective with MIC of 1200 µg/mL against *S. aureus* and *E. coli* with inhibition zones of 7 mm and 9 mm respectively. Moreover, human serum was effective against *E. coli* with a MIC of 200 µL/mL and the inhibition zone was 13 mm (Table 4). The efficacies of the three components surpassed that of the 14, 19, and 15 antimicrobials, which were ineffective against *S. aureus*, *E. coli*, and *P. aeruginosa* respectively (Table 4).

Table (4): Efficacy of silver nanoparticles, cerumen, and human serum on pathogenic bacterial agents from otitis externa

Tested substances	Inhibition zone diameters			Minimum inhibitory concentrations
	S. aureus	*E. coli*	*P. aeruginosa*	
Silver *nano-particles* (AgNPs)	-	30 mm	20 mm	650 µg/mL
Ear wax (cerumen)	7 mm	9 mm	-	1200 µg/mL
Human serum	-	13 mm	-	200 µL/mL

DISCUSSION

Otitis externa is considered one of the most prevalent disorders seen in otorhinolaryngology and is often seen in primary and pediatric care. It can range in severity from moderate to severe life-threatening malignant otitis externa infection [27]. Most of the otitis externa cases (more than 90%) result from bacterial infections. So we designed this study to focus on the most prevalent bacterial agents. **Guss and Ruckenstein** [18] result confirms our result about the rate of otitis externa caused by bacterial agents, which reached 65%. We found that 35% of the cases was not caused by bacterial infection which could be interpreted after **Wiegand *et al.*** [28], who stated that there are many factors that predispose for otitis externa other than bacterial infections. For example, anatomical causes stenosis, exostoses, and heavy hair coverage of the external auditory canal. Diseases of the skin as neurodermatitis, seborrhea, psoriasis, and eczema as well as the environmental factors such as increased humidity and temperature. Trauma, cerumen removal, and foreign bodies added to that systemic diseases as immunosuppression, metabolic diseases, and diabetes mellitus. Finally, lacking of cerumen production is considered as endogenous cause with other factors such as entrance of water and irritants in the canal.

The isolation rates of *S. aureus* and *P. aeruginosa* were 20 % and 30% respectively as confirmed by **Wiegand *et al.*** [28] who stated that *S. aureus* and *P. aeruginosa* were most commonly isolated from otitis externa with rates range of 11–34% and 22–62% respectively. Moreover, the isolation rate of *E. coli* was 15%, which was higher than **Getaneh *et al.*** [7]**,** who isolated it with a rate of 7.43%. They concluded that *S. aureus, proteus vulgaris, proteus mirabilis, E. coli*, and *P. aeruginosa* were the most prevalent bacteria causing infections to ear [7]. *S. aureus* was confirmed to be MERSA by PCR and showed susceptibility to amoxicillin and clavulanic acid, cefoperazone, ciprofloxacin, gentamycin, vancomycin, and imipenem with rates of 8 (40%), 18 (90%), 12 (60%), 12 (60%),

18 (90%), and 18 (90%) respectively. The efficacy of amoxicillin and clavulanic acid, ciprofloxacin, gentamycin, and imipenem were nearly similar to the recent results from Egypt [29]. The susceptibilities to cefoperazone and vancomycin are nearly similar to **Khatoon *et al.*** [27] and **Rajendran *et al.*** [30] respectively. The susceptibility of *E. coli* to imipenem was 14 (93.3%) higher than **Khatoon *et al.*** [29], who confirmed that *E. coli* from chronic otitis media was susceptible to imipenem with a rate of 8 (80%). *P. aeruginosa* isolates expressed resistance to 5 antimicrobials that were ciprofloxacin, imipenem, levofloxacin, penicillin G, and sulfamethoxazole/trimethoprim with rates of 27 (90%), 28 (93.3%), 28 (93.3%), 3 (1%) and 8 (26.7%) respectively. The sensitivity of *P. aeruginosa* to ciprofloxacin, imipenem, and levofloxacin is similar to **Allam *et al.*** [29]. The susceptibility to penicillin G and sulfamethoxazole/trimethoprim was higher than **Getaneh *et al.*** [7] and **Maclean *et al.*** [31] respectively. Based on these results, it was lucid that *S. aureus*, *E. coli*, and *P. aeruginosa* from otitis externa could be classified as multiple drug resistant (MDR) because they have MAR indices of 0.7, 0.95, and 0.75 respectively and showed resistance to three or more antimicrobial agents [32].

Although patients were advised to use the ichthammol glycerine packs, we prescribed oral amoxicillin and clavulanic acid antimicrobial pills with ciprofloxacin ear drops for treatment of cases that showed weak results of relief. The selected antimicrobial was after antimicrobial susceptibility testing. The results of antimicrobial susceptibility testing, which is part of the standard work of all clinical microbiological laboratories were used to determine the best therapeutic option for the treatment of bacterial infections. These findings shed light on local patterns of antimicrobial sensitivity, assisting physicians in selecting the most effective antibiotic medication [33]. However, there was scarce data on the distribution patterns of various types of antimicrobial resistance genes in *S. aureus* from otitis externa. The existence of methicillin resistance genes was highly prevalent in *S. aureus*, *mec*A1, A2, *mec* A1, and *mec*C confirmed that all *S. aureus* are of MRSA types. Added to that, the existence of *Bla*Z gene with *erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(G), and *erm*(Q) correlate with resistance to penicillin and erythromycin, respectively. Moreover, the presence of class 1 integron and cefotaxamases *CTX*-M and *CTX*-M-1 in *E. coli* correlated strongly with resistance to beta-lactams. Also the existence of *erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(G), and *erm*(Q) genes strongly correlated with resistance to erythromycin. The existence of class 1 integron and *CTX*-M in *P. aeruginosa* correlated with resistance to beta-lactams. While, the low rates of the macrolide resistance genes, *msr*E and *mph*E weakly correlated with erythromycin resistance, which suggests that resistance mechanisms are present. Inadequate antimicrobial treatment, drug misuse/improper selection, and patient noncompliance have led to changes in the antibiotic susceptibility of pathogenic bacteria as well as the development of resistance to routinely used antibiotics. Moreover, the active antimicrobial resistance genes play detrimental roles in the antimicrobial resistance process [9].

Antimicrobial resistance represents a highly important global challenge to successful bacterial illness therapy. It has been demonstrated that antibiotic resistance has a deleterious influence on both clinical and therapeutic results, resulting in everything from treatment failures and the need for more expensive and safer alternative drugs to higher morbidity and mortality rates, longer hospitalization, and higher healthcare costs. The search for novel antimicrobials remains a critical requirement in fighting bacterial diseases. Silver nanoparticles exhibited antimicrobial efficacy on *E. coli* and *P. aeruginosa* with inhibition zones of 30 mm and 20 mm, respectively and the MIC was 650µg/mL. This activity is confirmed by **Franci *et al.*** [8] who stated that silver has been used in ancient times as an antiseptic due to its antimicrobial effect against Gram-positive and negative bacterial agents. Recently, it gained much attraction for the synthesis of new antimicrobial classes. It changes the bacterial membrane permeability and drastically affects bacterial respiration [8].

The ear wax (cerumen) proved efficacy against *S. aureus* and *E. coli*. It was effective with inhibition zones of 7 mm and 9 mm respectively and the MIC was 1200 µg/mL. These results agree with **Okuda *et al.*** [34] who found that cerumen inhibited several types of microorganisms, such as *S. aureus*, *P. aeruginosa*, and numerous *E. coli* types. The low cerumen pH (6.1), and existence of lysozymes and saturated fatty acids all contribute to its antibacterial effects. The human serum was effective against *E. coli* only with an inhibition zone of 13 mm and the MIC was 200µL/mL. This result comes in agreement with **Alexander *et al.*** [35], who confirmed the efficacy of human serum against *E. coli* chi1776. This result also agrees with **Arzumanyan *et al.*** [17] who confirmed that the albumin component of (bovine serum, ovalbumin, and human serum) proved efficacy on *S. aureus*, *E. coli*, *Candida albicans*, and *Cryptococcus neoformans* cells [17]. The antimicrobial activity of human serum was regarded to its components as complement system proteins, immunoglobulins, antimicrobial peptides, and albumin [16, 17].

The limitations of this research are scarce data about the topic in various Egyptian localities, a low number of patients, and little information about risk factors. There was no isolation and assessment of fungal agents. Moreover, future studies required to evaluate the in vivo efficacy of silver nanoparticles, cerumen, and human serum on otitis externa bacterial causes are required.

CONCLUSIONS

The ear canal bacterial infections and the incidence of otitis externa are increasing as it reached 65% of the tested cases. *S. aureus*, *E. coli*, and *P. aeruginosa* were the highly prevalent causative bacterial agents of otitis externa. The selection of amoxicillin and clavulanic acid pills with ciprofloxacin ear drops for treatment of cases was after antimicrobial susceptibility testing. So continuous antimicrobial susceptibility testing is vital to detect effective antimicrobials against these pathogens. It also considered important in detecting the rates of antimicrobial resistance. Molecular investigation of the antimicrobial resistance genes could be vital tool for interpretation of the causes of resistance. Testing new antimicrobial agents is considered essential to override the drastic effects of antimicrobial resistance. The silver nanoparticles, cerumen, and human serum are promising in controlling these serious pathogens of otitis externa because these alternative antimicrobials have no resistance mechanisms inside the bacterial agents.

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