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SCREENING OF DIACETYL ACTIVITY AGAINST MDR BACTERIA INVOLVED IN OTITIS MEDIA PATIENTS IN BAGHDAD CITY

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ABSTRACT

The objective of this study was to investigate the antimicrobial susceptibility to diacetyl of antibiotic resistant bacteria isolated from otitis media. The highest antibiotic resistant bacteria was among Gram negative (73.4%, 66/90), followed by Gram positive, staphylococci both coagulase positive and coagulase negative (26.7%, 24/90). The highest resistance was recorded to 8 antibiotics, then 7 antibiotics. Fourteen isolates of *Pseudomonas aeruginosa* out of 35 isolates were resist to 8 antibiotics. Staphylococci exhibited resistance to high number of antibiotics, such as 11, 12 and 13 antibiotics. The effect of diacetyl at different concentrations (50, 100, 150, 200 µg/ml) were used using Muller-Hinton agar and well assay, 50µL was used in each well. Selected *P. aeruginosa* isolates resist to 9 and 10 antibiotics mostly exhibited no growth in plates (inhibition zone diameter ⩾70 mm). Staphylococci resist to 11, 12 and 13 antibiotics had no ability to stand the diacetyl activity even at low concentration and the inhibition zones were ⩾ 70 mm or no growth appeared.

KEYWORDS: Antibiotic resistance, Diacetyl, Otitis media.

INTRODUCTION

Otitis Media (OM) is the generic term for all types of inflammations of the middle ear. It is a common disease and is typically a persistent disease , often capable of causing severe destruction and irreversible effect with deafness , ie., associated with hearing impairment.[1] It is characterized by accumulation of mucus and fluids with in the middle ear with discharge and pus.[2,3,4] This infection is one for which antibiotics are prescribed routinely.[2,5]

Most studies have been found that the main causative agent is *Pseudomonas aeruginosa* followed by *Staphylococcus*, (mainly *S. aureus*), other Gram negative bacteria were also recorded, such as *Escherichia coli* , *Proteus* spp , *Klebsiella* spp, and others either in Iraq or worldwide.[2,6] Antibiotic resistance were developed extensively to antibiotics in use or not in used due to cross-resistance.[7] The overuse of all types of antibiotics, as well as using broad-spectrum antibiotics can lead to existence of antibiotic-resistant bacteria[8] and higher medical costs.

On the other hand probiotics represent a promise approach for dealing with pathogenic bacteria. These probiotics and especially Lactic acid bacteria (LAB) have several abilities to confer health conditions, they have been used to antagonize several food pathogens, either as a single strain or in combinations.[9,10,11,12] These bacteria produce several varieties of antimicrobial substances, it has been used against MRSA strains and ESBL bacteria successfully.[12]

Diacetyl having the formula ($CH_3COCOCH_3$) is one of the effective metabolites of LAB. It is low molecular compound[13], produced by different species of *Lactobacillus* and *Lactococcus* during cheese ripening and yogurt production[14], and also can be produced in cultivation Lab media by different LAB bacteria at amounts depending on growth conditions.[9,11,13]

Diacetyl (2,3-butanodione) is a volatile compound produced by different citrate utilizing LAB.[15] It is responsible for the typical flavor of butter and some cheese varieties, and is often used as flavoring compound of many food products , being considered a generally recognized as safe (GRAS) food ingredient and approved by FDA.[16] It showed a very strong activity towards different types of pathogenic bacteria[17,18], it had high activity against virulent MDR *Mycobacterium tuberculosis*.[19] . This study aimed to find out the activity of diacetyl towards multidrug resistant bacteria involved in otitis media

MATERIALS AND METHODS

Ear swabs from the discharge were taken from patients complaining of OM referred to Teaching Medical City Hospital / Baghdad and Children Welfare Teaching Hospital / Baghdad. The ages were between 3 months and 68 years, both males and females, the samples were collected for the last 6 months.

The swabs were cultured on different media.[20,21]. They were identified using standard biochemical tests and morphological characters.[21,22] Antibiogram for each isolate was done using Kisby-Bauer method using Muller-Hinton agar (OXOID, England), the NCCLS breakpoint was used for estimation of susceptibility and resistance[23,24], the antimicrobial agents used were: Tobramycin[TB 10 µg], Ciprofloxacin[Cip 5 µg], Gentamicin [GM 15 µg], Imipenem [Ipm 10 µg] , Ceftriaxone [CTR 30 µg] , Aztreonam[AT 30 µg], Amikacin [AK30 µg], Ticarcillin + Clavulanic acid [TCC 1.25+10 µg], Cefoperazone [CEP 75 µg], Azlocillin [AZT 75 µg] , Ceftazidime [CAZ 30 µg] , in addition to those agents , Penicillin [P 10 Unit] , Cloxacillin[CX 10 µg], Clindamycin [CD 10 µg] , Co-trimoxazole [CO 25 µg], Ampicillin [AM 10 µg], Oxacillin [OX 1 µg] and Erythromycin [E 10 µg] were added to Gram positive bacteria.[23,24]

Total of 90 isolates with multidrug resistance (MDR), resist to 3 antibiotics or more were selected for further studies.

The activity of Diacetyl: compound was tested using well assay[25,26], Muller Hinton agar plates were cultured with over –night growth from Nutrient broth of each isolates by using sterile swab so as to achieve confluent growth , the plates were allowed to dry , and by sterile cork borer ,five wells were done (5mm diameter). Four concentrations of diacetyl (Fluka) were prepared (50µg/ml, 100µgml, 150 µg/ml, and 200µg/ml) using warm sterile distilled water, 50µL from each concentration was applied to the wells of the cultured plates, distilled water was used as control. The plates were allowed to stand for one hour in refrigerator to allow diffusion then incubated for 24-48 hrs at 37°C. The diameter of growth inhibition zone was recorded [millimeter].

RESULTS AND DISCUSSION

Ninety MDR isolates were selected for testing the diacetyl activity, those were resistant to 3 -13 drugs/antibiotics. These isolates were distributed into Gram negative (73.4%) and Gram positive (26.7%). The highest number was for *P. aeruginosa* among the Gram negative. Followed by *E. coli*, *Proteus* spp, and then *Klebsiella*. Staphylococci either coagulase positive or negative were mostly equal , as shown in Fig 1.

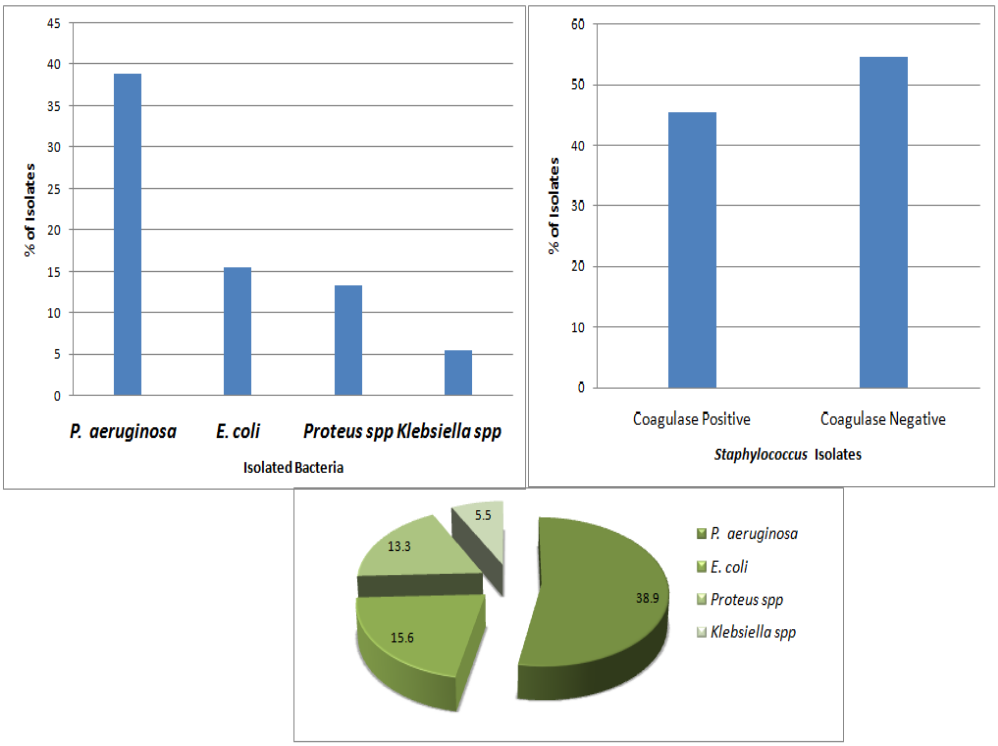

Fig 1: Types and percentages of antibiotic resistant isolates

Table 1 shows the number of isolates and the number of antibiotics they were resist to. The isolates were originally selected to be MDR as they were resist to 3 or more antibiotics, the results indicated that 20% of the total isolates were resist to 8 antibiotics , followed by those resist to 7 antibiotics (16.7%) , the lowest percentage recorded for 13 antibiotics (1.1%) for *Staphylococcus* spp , the rest were distributed between these extremes.

Table 1: Number of isolates resistant to different number of antibiotics.

Isolates	Total No. of isolates	No. of Antibiotics										
		No. of Resistant Isolates										
		3	4	5	6	7	8	9	10	11	12	13
P. aeruginosa	35	-	-	3	3	9	14	4	2	-	-	-
Escherichia coli	14	1	5	2	4	2	-	-	-	-	-	-
Proteus spp	12	-	4	4	3	1	-	-	-	-	-	-
Klebsiella spp	5	1	-	2	1	1	-	-	-	-	-	-
Staphylococcus spp	24	3	-	-	1	2	4	1	5	3	4	1
Total	90	5	9	11	12	15	18	5	7	3	4	1
%		5.5	10	12.2	13.3	16.7	20	5.5	7.8	3.4	4.5	1.1

\- No isolate recorded

Table 2 shows the inhibition zone of diacetyl effect on selected isolates which were resist to 5 antibiotics or more. *P. aeruginosa* which resist to 9 and 10 antibiotics were sensitive to diacetyl. These sometimes were assayed using one concentration / plate due to high effect, such situation was observed for other bacteria and was recommended to use plates with 150 mm in diameter rather than using the standard plates with 90 mm.[27,28,29]

Table 2: Effect of Diacetyl at different concentrations on different isolates resistant to number of antibiotics.

Isolate #	Number of Antibiotics	Concentration of Diacetyl [µg/ml], Diameter of inhibition zone [mm]			
P. aeruginosa		**50**	**100**	**150**	**200**
17	9	vs	vs	vs	vs**
12	9	vs	vs	vs	vs
13	9	vs	vs	vs	vs
22	9	vs	vs	vs	vs
26	10	34	40	vs	vs
31	10	vs	vs	vs	vs
Escherichia coli					
3	6	17	28	vs	vs
5	6	vs	vs	vs	vs
12	6	vs	vs	vs	vs
14	6	vs	vs	vs	vs
10	7	vs	vs	vs	vs
13	7	vs	vs	vs	vs
***Proteus* spp**					
2	6	vs	vs	vs	vs
7	6	vs	vs	vs	vs
10	6	vs	vs	vs	vs
12	7	vs	vs	vs	vs
***Klebsiella* spp**					
4	5	10	13	17	19
5	5	7	11	14	19
3	6	7	15	23	29
1	7	15	25	30	33
***Staphylococcus* spp**					
15	11	9	13	20	25
23	11	vs	vs	vs	vs
24	11	vs	vs	vs	vs
4	12	15	30	40	vs
5	12	vs	vs	vs	vs
21	12	vs	vs	vs	vs
7	12	vs	vs	vs	vs
11	13	vs	vs	vs	vs

**vs : very sensitive (Diameter ⩾70 mm) , OR No growth

All *Klebsiella* isolates were inferior in their susceptibility comparing to other group of bacteria, although they were still sensitive.

The results of Staphylococci are very interesting as that 8 isolates which were resist to 11, 12 and 13 antibiotics exhibited high susceptibility to diacetyl and the inhibition zone exceeds 70mm, and this led sometimes to use one concentration /plate as mentioned above . These data illustrate the higher recovery rate of multidrug resistance of isolates from OM. *P. aeruginosa* is the most common isolates in this study and other Iraqi studies[2,6,30,31], this might be due to primarily inhabitant of *Pseudomonas* in the environment, it is found in water, soils, and other environments throughout the world[32] and followed by Staphylococcus spp due to their different abilities of invasion.[33]

The situation is quite complex for resistance, once selected, may not go away after withdrawal of the antibiotic, i.e., the selective pressure. Although, this may be with a high metabolic cost for resistant bacteria to maintain the additional genetic materials associated with resistance[34], such as plasmids, transposons and others , but combinations of virulence factors and antibiotic resistance genes (which is considered sometimes as virulence factor), may make the pathogens better able to spread, colonies and invade a vulnerable patients.[34] Among the complications of antibiotic resistance is the presence of cross-resistance which could be carried out by plasmids.[33] In addition to the abuse of antibiotics , it is well known that high concentration of antimicrobial agents , the more rapid microorganisms are destroyed, though in some situations a low concentration is sufficient for treatment.[11]

The results of this study revealed that Staphylococci are resistant to large number of antibiotics , this could be due to cross-resistance which can be confer by *cfr* gene and its product methyltransferases that modified the PTC (Peptidyl Transferase Center), which is a target of many antibiotics, this *cfr* gene is primarily bound in plasmid facilities the horizontal transfer between staphylococci.[35,36,37] In addition most staphylococci harbor Staphylococcal Cassette Chromosome mec (SCCmec), which help this group to resist many antibiotics mainly methicillin.[38,39] It has been reported that the effect of diacetyl on Gram negative is higher than Gram positive bacteria[16,40,41] and to less extent to yeasts and fungi.[42] It has been suggested that diacetyl might be exerted its effect by inactivation of arginine utilization through its reaction with arginine-binding proteins.[43] But on the other hand diacetyl is lipid soluble substance and this will allow it to penetrate the outer

membrane of Gram negative and exerts more activity, and could penetrate the cell membrane of Gram positive and lead to leakage of cytoplasmic components, however, this needs more studies for confirmation.

To face the horrible situation of antibiotic resistance, a new class of drugs are needed, these could target the virulence factors rather than the bacteria cells which could elevate the development of resistance, such as inhibiting the biofilm formation or any other virulent factors.[44] This could be facilitated by using Computer Aided Drug Design (CADD).[45]

CONCLUSION

It is obvious that diacetyl has a very high antimicrobial potential, in parallel to its safety , it is working at pH(s) less than or equal to 7.0[46], so, it could be formulated to be use at least for external use and could be used for treatment of OM. It was also suggested that probiotic metabolites including the diacetyl to be a feasible option for chemotherapy of fungal infections[42], given the drug resistance exhibited to antifungal agents currently in treatment of ear infections.

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