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Uronic Acid andSome virulance factors of the Pathogenic Bacteria isolated from Patients with Otitis Media in Hilla City, an in vitro study, Iraq

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ABSTRACT

Background: Otitis media is inflammation of the middle ear, it constitutes the most common respiratory tract infection of infancy and early childhood**Objectives:** Isolate and identify the common bacterial pathogenes associated with otitis media, to examine some virulant factors such as (capsule, hemolysin, siderphores, extracelluar proteases, and CFA). And Detection of uronic acid from ear discharge.**Results:** Bacterial cultures were positive in (95%) patients versus (5%) revealed negative bacterial culture. The most common type of bacterial isolated was *Moraxella catarrhalis* (20.83%), followed by *Pseudomonas aeruginosa* (20%). Besides, uronic acid was detected in most samples of ear discharge. The virulence factors were detected and found to be capsule, colonization factor antigens, hemolysin, siderophores, and extracellular proteases.**Conclusion**: most common bacteria isolated are *M.catarrhalis*, *P.aeruginosa*,*S.pneumoniae*and *S. aureus.*All isolates have the ability to possess more than one virulence factors such as capsule, siderophores, hemolysin, extracellular protease, and adherence factors, which qualified otitis media. The concentration of uronic acid increased with large size of capsular polysaccharides around the bacteria

INTRODUCTION

Inflammation of the ear is one of the most common illnesses inchildren. Otitis media is an inflammatory disease of the mucosallining of the middle ear(Elmanama*et al*., 2014). Otitis media may be acute,or chronic suppurative type.Children are mostly affected with acute type,while adults are mostly affected with chronic suppurative types(Papavasileiou*et al*.,2009 andHamada *et al*.,2012).The infection of otitis media might result from viral or bacterial agents, however many complications were reported that may persist in some individuals in to adult years(Little*et al*.,2006). The bacteriologic agents associoted with otitis media are; *S.peumoniae* ,non typable *H. influneza,* and *M. catarrhalis* are most common bacterial of acute OM. (de Vries *et al*., 2009), *P. aeruginosa, S.aureus, and Proteus spp.* are predominant pathogen of chronic suppurative otitis media,and also it was seen that non spore forming anarebes such as *Bacteroides* spp can be isolated from exudates obtained from patients with chronic otitis media AL- Dahhan*et al*., (2005) and Broides*et al*., (2009). Pathogenic bacteria have the ability to produce several types of virulence factors associated with their pathogencity and major role in the causation of infection in otitis media, the main virulence factors are capsule ,hemolysine, siderophores, proteases,and CFA. Otitis media in Iraq in all types are recorded and the prevelence of it differs according to the types of otitis media, and also patients location , sex and occupation. Thisstudyaimsto**;** isolate and identify the common bacterial pathogenes associated with otitis media, to examine some virulant factors such as (capsule, hemolysin, siderphores, extracelluar proteases, and CFA). And Detection of uronic acid from ear discharge.

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MATERIALS AND METHODS

1 Patients:

Discharge samples of 100 patients with O.M and 50 healthy as a control group were collected.

2-Methods:

Collection of Specimens:

Isolation and all biochemical tests for diagnosis of bacteria ofO.M was done according to MacFaddin, (2000) and Forbes*et al.*,(2007)

Virulence Factor Tests:

1- Capsule Stain Test (Hiss's Method). According to Cruickshank, *et al.*, (1975).
2- Blood Hemolysis Production Test.According to Cowan, (1985).
3- Siderophores Production Test. According to Nassif, *et al*., (1989).
4- Extracellular Protease Production Test: According to (Piret, *et al.*,(1983).
5- Haemagglutination Test (HA). According to (Francis, *et al*., (2002).
6- Extraction the polysaccharide and detection from colonic acid. According toHindi, (2008)

Results:

1. Bacterial Isolates from otitis media:

Ninty five samples with +ve culture, whereas 5 samples with no bacterial A total of 120 bacterial isolates were obtained from the 95 samples collected. From the results it was shown that Grams negative bacteria constitutes 57.5% (69/120) from the total isolates and were considered the predominant aetiological agent of OM compared to gram positive bacteria which constitute 42.5%(51/120 isolates).

2.Pathogenicity of bacteria in Otitis Media:

Sixty eight cases of OM were caused by single bacterial.where *P.aeruginosa* constitutive (16/68), *M.catarrhalis* (14/68) were predominant in single causative microorganism followed by *S.aureus* (11/68), *S.pneumoniae* (9/68).However, each of the following bacteria *K. Pneumoniae*, *S.epidermidis*, *Acintobacter spp* and *Proteus spp* constitutive (4/68). The mixed bacterial types (52) were related to two different genus and species), this might describe a cooperation between these different species in disease processes. *Sterpt.pneumoniae* (14) isolates were predominant in mixed infection .This in the finding that *S.pneumoniae* produce protease and also contain capsule. The capsule resistance for phagocytosis and the protease is effective against IgA give support for other bacteria causes OM such as *S.aerues, P.aeruginosa* ,and *M.catarrhalis.*

P.aeruginosa are producing large number of extracellular products such as alkaline protease, elastases and exotoxin A which can cleave IgA lead to inhibit the function of the immune system of the cells, this give support for other bacteria causes OM such as *S.aurues, Proteus spp* ,and *E.coli.*

K.pneumoniae contain thick capsular polysaccharide which considered resistant tophagocytosis, this support *M.catarrhalis* and *E.coli* for causes OM.

Dissucsion:

Pathogenicity of bacteria in Otitis Media:

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Anaerobic bacteria were mostly isolated in mixed culture with facultative anaerobic bacteria (*S.pyogenes*) and represented in one case.This finding agrees with Ibekwe*et al*.,(1997) who found those anaerobes represent about 0.9% of all isolates. This pattern of mixed culture betweenaerobic and anaerobic bacteria may be attributed to the synergistic relationship between aerobes and anaerobes,in which the aerobic bacteria removes oxygen, produces substances that lower the potential of the tissues, or provide nutrients that are necessary for

the proliferation of oxygen the anaerobic pathogens, whereas anaerobic bacteria alone does not usuallly establish themselves in a suppurative process.

Virulance Factors:

Most strains of *M. catarrhalis* that adherence were mediated through fimbriae or pili enhance attachment to host cells and resistance to phagocytosis to provide a way for the bacterium to evade the hosts immune response .Capsule was detected in some isolated bacteria by using Hiss methods. The results in **tab.1** reveal that all isolates of *M. catarrhalis* which were isolated from patients with bacterial OM did not possess the polysaccharide capsule.This result is similar to the result observed by Ahmed*et al*.,(1991) who had pointed out that *M. catarrhalis* could not possess the polysaccharide capsule. All *M. catarrhalis* isolates were found not capable of producing the extracellular hemolysin. Only 4 isolates of *M. catarrhalis* were able to produce siderophores.

Furthermore, it was known that bacteria which were able to produce siderphores have no ability to produce hemolysin, so bacteria need only one mechanism for obtaining iron. Iron can increase disease risk by functioning as a readily a vaible essential nutrient for invading microbial and neoplastic cell,to survive and replicate in hosts, microbial pathogens must acquired host iron, this identical with that result obtained by Ibekwe*et al*.,(1997) and Arroll**,** (2005). The ability of *M. catarrhalis* to produce extracellular proteases was investigated and it was found that only 2(20%) isolates of *M. catarrhalis* were able to produce extracellular proteases. *M. catarrhalis* can produce proteases when reach mucosal surfaces may often encounter secretory IgA, which could inhibit their adherence and growth on epithelium, the bacteria that cause disease on these mucosal surface were able to evade the action of secretory antibody by producing IgA proteases, that inactivate IgA antibody and this enzyme play role in colonization of mucosal surface. The bacteria produce several protease which have the capacity to degrade host proteins releasing amino acid as nutrients and may degrade proteins such as IgA which are involved in host defense, and may also be involved in host tissue damage(AlTimimi,. 2004 andAlUbaydi,2006).*P. aeruginosa* isolates virulence factors are shown in **tab. 2**,the isolates were tested for their abilities to produce adherence factors , and it was found that 5 bacterial isolates were able to produce adherence factors, this may be due to the presence of fimbriae, the fimbriae of *P.aeruginosa* will adhere to epithelial cells of the upper respiratory tract and causes OM, this identical to result obtained by Mouricout,(1997). Also, it was found that 60% of *P. aeruginosa* isolateswere possessing the polysaccharide capsule which surrounded the bacterial cell,capsular polysaccharide can protect *P. aeruginosa* against the ciliary action of the respiratory tract and complement mediated lysis ,this agree to the result mentioned byGoodierandLodei, (2000).

Itwas found that only 3 isolates of *P. aeruginosa* were able to produce extracellular hemolysin. *P. aeruginosa* produce two hemolysin, it appears to be cytotoxic for most eukaryotic cells, hemolysins conttribute to invasion through their cytotoxic effects on eukaryotic cells.*P. aeruginosa* isolates were also tested for their abilities to siderophores synthesis.The results showed that all isolates of *P. aeruginosa* were able to produce siderophores,and also,it was shown that only 3 isolates of *P. aeruginosa* can produce extracellular proteases. *P. aeruginosa* could produce of large number of extracellular proteases such as alkalin protease,elastases , and exotoxin A which can cleave IgA which then lead to inhibit the function of the cells of the immune system , thus *P.aeruginosa* is resistant for phagocytosis and opsoization ,this agree with that result mentioned by StenforsandRaisanan,(1992), also found that toxin A and elastase inhibit protien synthesis in macrophage of mice thus directly effect phagocytosis.

The ability of *S. pneumoniae* isolates to produce some virulence factors were investigated. *S. pneumoniae* isolates were tested for their abilities to produce colonization factors antigen as shown in **tab. 3**,and it was found that all isolates were able to produce colonization factors antigen in the presence human blood (group A).

Further more ,it was found that only 4 isolates from 6 isolates of *S.pneumoniae* 66.6% had capsular polysaccharide,The capsular polysaccharide is very essential virulence factors through protecting the organisms from complement activation and phagocytosis mediated destruction.

Additionally, the detection of hemolysin in blood agar was also studied. It was found that all *S. pneumoniae* isolates produced hemolysin, and the type of hemolysis was Alpha-hemolysis due to the presence of a green zone of hemolysis that surrounded the bacterial colonies formed on blood agar. *S. pneumoniae* produced an Alpha-hemolysin that reduced hemoglobin (red) to methemoglobin (green), caused a greenish zone to surround the colony ,The same results were obtained by FacklamandPigott,(1994).The function of hemolysin is to provide the microorganism with iron and it makes the bacteria unable to produce any factor for obtaining the iron from environment.Moreover,the ability of *Strep. pneumoniae* to siderophores synthesis was studied, and it was found that all isolates of *S. pneumoniae* could not produce siderophores or iron-chelating factors under low-iron conditions and could use either hemin or hemoglobin as a sole source for the required iron. Thefindingagree with that obtained by Tai, *et al.*,(1993). The ability of *S. pneumoniae* to produce extracellular proteases was investigated and it was found that only one isolates of *S. pneumoniae* were able to produce extracellular proteases. *S. pneumoniae* can produce IgA1proteases, and these proteases are of the serine type

which enables *S. pneumoniae* to evade the protective functions of the principal Ig isotype of the upper respiratory tract,these proteases degrade human Ig A1, as well as on the balance between secreted and cell-associated forms of the enzyme. These results were comparable to those obtained by Poulsen *et al.,* (1996) and Reinholdt and Kilian,(1997).

The ability of *S.aureus*VFs are shown in **tab. 4**. *S. aureus* isolates were tested for their abilities to produce colonization factors antigen , and it was noticed that 4 bacterial isolates could produce CFA. Several studies had shown that *S.aureus* strains could adhere to host tissues throgh teichoic acid,this acid was one of the most important component in bacterial cell wall. However, the ability to produce additional adherence factors would help the bacteria to colonize various tissues.Moreover, some strainsof *S. aureus* 10% had shown to possess the polysaccharide capsule which was an important component in the pathogenesis, and enhances bacterial virulence by modulate *S. aureus* adherence to endothelial surface in vitro, animal studies suggest that it also promotes bacterial colonization and persistence on mucosal surfaces, this agree with result mentioned by Nair*et al*,(2000).

All *S. aureus* isolateshad the ability to produce the hemolysin, and the type of hemolysis was Beta-hemolysis due to the presence of a clear zone of hemolysis around the bacterial colonies formed on human blood agar, this agree with result mentioned by Dinges,*et al.*,(2000). *S. aureus*was also tested for it's ability to siderophores synthesis, and the results showed that all isolates of *S. aureus*were not able to produce siderophores. The production of hemolysin would make the bacteria does not need the production of siderophore for obtaining iron because the former would help it to gain the iron.Itwas found that 2 isolates of *S. aureus* were able to produce extracellular proteases, but other bacterial isolates, 8 isolates could not produce this enzyme. The production of extracellular protease varied considerably among clinical isolates of *S. aureus*, and the presence of the major protease genes, in all the protease-negative strains analyzed suggested that the lack of protease production was due to some regulating host cell factors. This identical with result mentioned by Karlsson and Arvidson, (2002).

Regarding to *K. pneumoniae*VFs are shown in **tab.5**. The isolates were tested for their abilities to produce colonization factors antigen, and it was found that 3 of *K.pneumoniae* isolates were able to produce CFA due to the positive reaction with human RBC (groupA). *K. pneumoniae* strains had the ability to possess type 3 pili, which was an important factor in adherence of this type of bacteria to mucous of respiratory tracts. Theseresults was identical with the results obtained by Venegas*et al.,* (1995) and Hornick*et al.,* (1999).All*K. pneumoniae* isolates were also possessing the polysaccharide capsule which surround the bacterial cell. This result agrees with the result obtained by Podschunand Ulmann, (1998) who had pointed that the diagnostic feature for *K. pneumoniae* is capsular polysaccharide and it's essential virulence factor for *K. pneumoniae*. The results also showed that all *K. pneumoniae* isolates could not produce hemolysin extracellulary when cultured on blood agar, and the type of hemolysis was gamma hemolysis because there was no hemolysis present on blood agar.*Klebsiella pneumoniae* isolates were also tested for their abilities to siderophores synthesis.The results showed that all isolates of *K. pneumoniae* were able to produce siderophores, all *Klebsiella*strains have the ability to possess siderophores system and *K.pneumoniae* depend on this system for the uptake of iron which may be considered an essential factor in bacterial growth. Thus, production siderophores may give access to both sources of iron, resulting in enhanced growth in the host , from the results obtained in this study it was shown that the bacteria can only produce either hemolysin or siderophores not the both .

Also it was found that 3 isolates of *K. pneumoniae* could produce extracellular proteases *K. pneumoniae*. Proteolytic enzymes were very important factors in which the bacteria can degrade protein and then causes penetration to the tissues, this identical with that result obtained byAl Dahhan, (2001).

Detection of Uronic acid:

Uronic acid in 22 samples of ear discharges were selected on the basis of the presence of mucoid discharge and investigated by using colorimetric method **tab. 6**. It was found that uronic acid was detected in most samples of ear effusion taken from *K. pneumoniae*, *S. pneumoniae* and *P.aeruginosa* isolates. This may be attributed to the possession of these bacteria to capsular polysaccharides, which may be secreted extracellulary as a uronic acid.

The size of capsules also may have effect on the mount of the uronic acid produced the bacteria. So, the bacteria with small capsule have a little amount of production of this acid .

Uronic acid was not detected with the isolates of *M. catarrhalis* when taken the ear sample (without discharge) as a controling group. This may be attributed to *M. catarrhalis* had not capsular polysaccharides to produce effusion (we conclude that bacterial isolated which produce $\leq$ 10 Microgram/ml concentration of uronic acid considered less amount of uronic acid and not capable to produce capsule). Uronic acid can protect the bacteria from secretory IgA produced by mucosal surface of upper respiratory tract infection,therefore uronic acid important in pathogencity of bacteria that causes OM infection and ear discharge. *P.aeruginosa* were mainaly isolated from chronic OM infection , and were characterized by mucoid effusion . This effusion were may be due to alginate which is the main character for the production of uronic acid, this agree with result

mentionedby Stevens*et al*.,(1984).*P.aeruginosa* isolated from patients wih OM had a mucoid colony morphology due to overproduction of the alginate, which contribute to the persistence of bacteria in patients this agree with mentioned by JainandOhman (2002)andLee, (2003). The control of the production of uronic acid by bacteria in ear effusion by a regulator genes, these indicated that genes responsible from uronic acid production found on bacterial chromosome or plasmid, those genes role play important in increased the acid production.

Table 1: Type of virulence factors detected in *M. catarrhalis*

No.of Isolates	CFA	Capsule	Heamolysin	Sidrophores	Extracellular proteases
1	+	-	-	-	+
2	+	-	-	-	-
3	+	-	-	-	+
4	-	-	-	-	-
5	+	-	-	+	-
6	+	-	-	+	-
7	+	-	-	-	-
8	+	-	-	-	-
9	+	-	-	+	-
10	-	-	-	+	-
Total	80%	0	0	40%	20%

Table 2: Type of virulence factors detected in *P. aeruginosa*

No.of Isolates	CFA	Capsule	Heamolysin	Sidrophores	Extracellular Proteases
1	-	-	+	+	-
2	+	-	-	+	-
3	+	+	-	+	+
4	-	+	-	+	-
5	+	+	+	+	-
6	-	-	-	+	+
7	-	+	-	+	+
8	+	+	+	+	-
9	+	+	-	+	-
10	-	-	-	+	-
Total	50%	60%	30%	100%	30%

Table 3: Type of virulence factors detected in *S. pneumoniae*

No.of Isolates	CFA	capsule	Heamolysin	Sidrophores	Extracellular Proteases
1	+	-	+	-	-
2	+	-	+	-	+
3	+	+	+	-	-
4	+	+	+	-	-
5	+	+	+	-	-
6	+	+	+	-	-
Total	100%	66.6%	100%	0	16.6%

Table 4: Type of virulence factors detected in *S. aureus*

No.of Isolates	CFA	capsule	Heamolysin	Sidrophores	Extracellular proteases
1	+	-	+	-	-
2	+	-	+	-	+
3	-	-	+	-	+
4	-	-	+	-	-
5	+	+	+	-	-
6	-	-	+	-	-
7	-	-	+	-	-

8	-	-	+	-	-
9	+	-	+	-	-
10	-	-	+	-	-
Total	40%	10%	100%	0	20%

Table 5: Type of virulence factors detected in *K. pneumoniae*

No.of Isolates	CFA	capsule	Heamolysin	Sidrophores	Extracellular Proteases
1	+	+	-	+	+
2	+	+	-	+	+
3	-	+	-	+	+
4	+	+	-	+	-
Total	75%	100%	0	100%	75%

Table 6: Concentration of uronic acid in isolated bacteria

NO.	ISOLATES	CAPSULES	CONCENTRATION OF UAμ g/ML
1	*S. pneumoniae*	+	22
2		+	29
3		+	15
4		+	28.9
5		+	27.6
6		+	41.5
7		+	31.4
8		+	75
1	*P.aeruginosa*	+	15
2		-	4.4
3		+	15
4		+	36.6
5		+	56.2
6		+	33
7		-	5.4
1	*K. pneumoniae*	+	61.2
2		+	18.4
3		+	16.4
1	*S. aureus*	+	48
2		-	8.5
3		+	17.5
1	*M. catarrhalis*	-	4.4

Control ≤ 10

Conclusions:

According to this study, we can conclude the following; The types of bacteria which causes otitis media from the most to least common are *M.catarrhalis*, *P.aeruginosa* ,*S.pneumoniae*and *S. aureus.* All bacteria isolates in this study have the ability to possess more than one VFs such as capsule, siderophors, hemolysin, extracellular protease, and adherence factors to produce O.M. The high concentration of uronic acid increased with large size of capsular polysaccharides arounded the colonies.

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