Characterization of *Aeromonas hydrophila* Isolated from Patients with Diarrhea

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خصائص بكتريا *Aeromonas hydrophila* المعزولة من مرضى الأسهال

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Abstract:

Aeromonas hydrophila was isolated from 12 (5.3%) of 225 patients with acute gastroenteritis.The present study was determined the virulence of *A. hydrophila* obtained from patients with diarrhea, among 12 *A. hydrophila* isolates obtained from stool, 83.3%, 91.6%, 83.3%, 75%, 75%, 50% produced β-haemolysin, protease ,phospholipase, DNase, slime(viscous glycoconjugate material), and have the adhesion, respectively. The isolates were tested for their susceptibility to 21 antibiotics, high level of resistance was observed to Penicillin, Carbenicillin, Amoxicillin/Clavulanic acid, followed by high level of susceptibility towards Azithromycin 100%, Ofloxacin 100%, Meropenem 100%, Cefepime 91%, Streptomycin 91%, Aztreonam 91%. Minimum inhibitory concentrations (MICs) of *A.hydrophila* isolates to certain antibiotics, azithromycin, ofloxacin, chloramphenicol and cefepime, was done by agar dilution method, azithromycin and ofloxacin have the lowest MICs 1-32µg/ml, 1-128µg/ml, respectively. Exposure of *A. hydrophila* to sub-MICs of such antibiotics azithromycin , ofloxacin, chloramphenicol and cefepime, reduced the percentage of haemolysin, protease ,phospholipase ,DNase and slime production.

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الخلاصة:

عزلت بكتريا *Aeromonas hydrophila* بنسبة 12 (5.3%) من 225 مريض بألتهاب المعدة والامعاء الحاد. حددت هذه الدراسة ضراوة بكتريا الـ *A. hydrophila* واللتي حصل عليها من مرضى الاسهال, من ضمن 12 عزلة من *A.hydrophila* تم الحصول عليها من الغائط, 83.3%, 91.6%, 83.3%, 75%, 75%, 50% منتجة للـ - βhaemolysin, protease, phospholipase, DNase, slime (viscous glycoconjugate material) والقابلية على الالتصاق, على التوالي. لقد تم اختبار حساسية العزلات للـ21 مضاد حيوي, مستوى عالي من المقاومة لوحظ للـ Penicillin, الـ Carpenicillin والـAmoxicillinClavulanic acid, بينما اظهرت حساسية عالية تجاه الـ Azithromycin 100% , Ofloxacin 100% , Meropenem 100%, Cefepime 91% , Streptomycin 91 %, Aztreonam 91%. تم تحديد اقل تركيز مثبط لعزلات *A. hydrophila* من المضادات الحيوية , ofloxacin , chloramphenicol azithromycinوالـ cefepime بأستخدام طريقة الانتشار الطبقي, اذ وجد ان الـ azithromycin والـ ofloxacin لها القدرة على تثبيط البكتريا بتراكيز قليلة (1-32مايكروغرام/مللتر) و(1-128مايكروغرام/مللتر) على التوالي, اظهرت العزلات عند تعرضها للتراكيز تحت التراكيز المثبطة لمضادات azithromycin, ofloxacin, chloramphenicol والـ cefepime اختزالا في نسبة تحليلها للدم وانتاجها للـ protease , phospholipase, DNase و المادة المخاطية .

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Key words: *Aeromonas hydrophila* , virulence factors , antibiotic susceptibility.

Introduction:

Aeromonas hydrophila is an oxidase-positive, facultative anaerobic, Gram-negative bacillus of the *Vibrionaceae* family, *A.hydrophila* is wide spread in nature and is usually found in fresh water, brackish water, moist oil and non-fecal organic material, it is the most common human pathogenic species of the *Aeromonas* genus (68%),   according to a previous epidemiological report [1].

Known risk factors for disease in humans include drinking or swimming in contaminated water and also ingestion of contaminated food, direct contact with contaminated animals and using ineffective antimicrobial agents are the other risk factors. Foods of animal origin and contaminated animals may play an important role in the transmission of the aeromonad from food or animals to humans and animal feces appear to be the major source of contamination of foods [2].

Human Infection caused by *Aeromonas* spp. include acute gastrointestinal illness ,soft tissue infections ,sepsis ,frequently in association with malignancies and cirrhosis ,and other miscellaneous infections ,including pneumonia ,meningitis, endocarditis ,septic arthritis and osteomylitis , *Aeromonas* can also isolated from the   stools of asymptomatic indivduals [3].

Virulence of *Aeromonas* spp. is multifactorial and not completely understood,   *Aeromonas* spp. have been reported to elaborate exotoxins (haemolysins, cytotoxins, enterotoxins), hemagglutinins, adhesins, several hydrolytic enzymes, and invade tissue in culture[4].

Although infections due to *Aeromonas* may be self-limiting, treatment with antibiotics is generally necessary to curb the progression and persistence of the disease, particularly in vulnerable groups, such as the young, elderly, and immunocompromised individuals, the growing antibiotic resistance of pathogenic bacteria worldwide is a compounding factor for the effective management of bacterial infections, an increase in antibiotic resistance of the genus *Aeromonas*, particularly to antibiotics, has been reported [5] .

Although there are certain alternatives to the use of antimicrobial agents, such as vaccination, immunostimulants or probiotics, antimicrobial chemotherapy still represents the method of choice for control of most bacterial infections in human[6]. The main problem involving the use of antibiotics against *Aeromonas* infections is the development of resistance by these bacteria generally related to the presence of plasmids [7]. The present study evaluated the prevalence pathogenic indices, antibiotics susceptibility profiles, minimum inhibitory concentrations (MICs) to antibiotics and the effect of sub-MICs of some antibiotics to pathogenic indices against *A.hydrophila* isolated from diarrheic stool samples of patients with gastroenteritis.

Materials and Methods:

The study was carried out in Microbiology Department,University of Al-mustansiryah from October 2008–April 2009. Diarrheic stool samples were collected from patients attending the Al-Yarmok Hospital/ Teaching Laboratories Department / Ministry of Health.

Fecal specimen:

In total, 225 stool samples were collected and investigated for *Aeromonas* spp. The specimens were cultured using the method as previously described [5], briefly, freshly-collected stool specimens were plated onto MacConkey agar (Oxoid), xylose deoxycholate citrate agar (XDCA) (Difco) and sheep blood agar containing 10 µg/ml ampicillin (Hi-Media) and incubated at 35°C for 18-24 h, after which non-lactose-fermenting colonies on MacConkey agar and non-xylose-fermenting colonies on XDCA were screened for the production of oxidase. Oxidase-positive colonies were subcultured onto nutrient agar plates (Oxoid). To discriminate *A.hydrophila* Kligler

test (strains yielding alkaline slant and an acid butt reaction after 24 h at 37°C demonstrated the presence of *A. hydrophila*)[8].

Determination of virulence factors:

Haemolysin assay: Haemolysin production was determined using blood agar plates (Hi-Media) and also cell free haemolytic method [9].

Protease assay: Protease activity was assayed by spreading *Aeromonas* isolates on nutrient agar containing 10% skim milk (Difco), after incubation for up to 24 h at 37°C, protease production was shown by the formation of a clear zone caused by casein degradation [10].

Phospholipase assay: overnight cultures of *A.hydrophila* screened for their extracellular phospholipase activity by growing them on egg yolk agar (Oxoid) [11]. Egg yolk was aseptically separated from commercially bought eggs (Steger's Fresh, Titusville, Md.), mixed with sterile phosphate buffered saline (PBS)(Dulbecco A, Oxoid) , and then mixed with sterile nutrient agar with 1% NaCl at 50°C in a ratio of 1ml of yolk suspension per 20 ml plate ,colonies expressing phospholipase activity at 24h [12]. Positive colonies were distinguished by the presence of precipitation zone of degraded egg yolk surrounding the colonies [11].

DNase assay: extracellular nucleases (DNases) were determined on DNase agar plates (Difco) with 0.005% toludine blue, five microliters of each suspension was streaked onto the plates and incubated at 37°C for 24 h. a pink halo around the colonies indicated nuclease activity [13].

Slime test: brain heart infusion agar plates (Hi-Media) were prepared containing 0.8 g/l Congo red, *A.hydrophila* isolates were inoculated onto the surface of the medium and the plates were incubated at 30°C for 24 h. Bacteria producing slime appeared as black colonies, whereas, non-slime producers remained non pigmented [9].

Adhesive properties: human uro-epithelial cells were obtained from early-morning first-stream urine samples of five healthy females (age range 23-29 years) with regular menstrual cycles, preparation of uro-epithelial cells, cells were harvested from the urine immediately after collection by centrifugation (5000 rpm) (Centrimax), washed twice in (PBS) pH 7.3 and suspended in PBS at a concentration of l0 cells/ml, estimated by counting the cell suspension in a modified Fuchs-Rosenthal counting chamber [14] .

Adhesion test: one-ml volumes each of bacterial suspension (l0^8 bacteria/ml) and uro-epithelial cells (10^5/ml) were mixed and incubated at 37°C for 1 h. in an orbital incubator at 60 rpm, epithelial cells were recovered by centrifugation at 90 rpm and unattached bacteria removed by washing twice in PBS, the epithelial cell preparation was then fixed by air drying to a glass slide and stained by Gram's method, the numbers of bacteria adhering to each cell were seen by light microscopy [14].

Testing of antibiotic susceptibility:

Antibiotic susceptibility of the isolates was determined using the disc-agar diffusion technique, several antibiotics were tested and were obtained from (bioanalyse), these included: piperacillin (100μg), cephalothin (30μg), trimethoprim/sulfamethoxazole (25μg), meropenem (10μg), clarithromycin (15μg), chloramphenicol (10μg), amoxicillin/clavulanic acid (30 μg), kanamycin (30μg), cefixime (5 μg), cefoxitin (30 μg), rifampin (5μg), sulbactam/ampicillin (20μg), ceftazidime (30 μg),ofloxacin (5 μg), aztreonam (30 μg), azithromycin (15μg), cefepime (30 μg), carbenicillin (100μg), ceftizoxime (30μg), streptomycin (10μg), penicillin (10μg). Briefly pure colonies of each bacterial strain were inoculated into 2 ml of sterile Mueller- Hinton broth (Difco) in bijou bottles and incubated at 37 °C for six hours, the turbidity was adjusted to match a 0.5 McFarland turbidity standard, a sterile cotton-tipped swab was dipped into the standardized bacterial suspension, and the swab was rotated against the wall of the tube above the liquid level to

remove excess inoculums, the inoculums was swabbed on the entire surface of a Mueller-Hinton agar plate (Difco), the automatic disc dispenser, adjusted to dispense six antibiotic discs, was applied on the surface of the agar, and the plates were incubated at37 °C for 18 h. (CLSI,2009) [15].

Minimum inhibitory concentrations (MICs):
MICs of selected antimicrobial agents were determined, using an agar dilution method [16]. Mueller-Hinton agar was the basic medium, double dilutions of antibacterial agent stock solutions were incorporated into the agar plates, with final concentrations ranging from 0.5 to 1024 µg/ml, the isolates were cultured overnight in tryptic soy broth (Sigma) at 28°C, and cultures were adjusted to an optical density of a 0.5 McFarland standard, diluted 1:10 in PBS, and applied as 1 µl droplets to the plates, every test was run in duplicate on freshly prepared agar plates, after 24 h of incubation at 35°C the MIC for each isolate was determined as the lowest concentration of the antimicrobial agent able to inhibit bacterial growth [6].

This study was undertaken to investigate the effect of sub-MICs of azithromycin, ofloxacin, chloramphenicol and cefepime, on haemolysin, protease, phospholipase, DNase and slime production of isolates.

Results :
Of the total of 225 patients with acute gastroenteritis, 12(5.3%) *A.hydrophila* isolate were detected. The isolation rate of *A.hydrophila* were higher among male than female (66.6%), (33.4%), respectively .*A.hydrophila* was most commonly isolated from the age group of 6 month-10 years (66.6%) (Table-1).

Table-1: Distribution of infection according to the age and sex

Age group (Year)	No. 0f diarrhea patients		No. *A. hydrophila* isolates	Gender	
				Male	Female
6 month-10 year	Male	92	8	5	3
	Female	66			
10 -20	Male	11	1	1	-
	Female	5			
20 -30	Male	8		-	-
	Female	5			
30 -40	Male	9			-
	Female	6			
40 -50	Male	5	2	1	1
	Female	4			
50 -60	Male	6	1	1	-
	Female	5			
60 -70	Male	2	-	-	-
	Female	1			
Total		225	12	8	4

A.hydrophila demonstrated β-haemolytic activity on the human red blood cells agar (83.3%), all the isolates were tested for protease ,phospholipase, DNase, slime production, protease activity detected in (91.6%) isolates, phospholipase (83.3%), DNase (75%), slime producer (75%), whereas, adhesion ability showed in (50%) isolates (Table-2).

Table-2: Percentage of virulence factors of *Aeromonas hydrophila* isolated from diarrheic stool

Virulence factors	*Aeromonas hydrophila* %
β- Haemolytic activity	83.3
Protease production	91.6
Phospholipase production	83.3
DNase production	75
Slime production	75
Adhesion ability	50

Results obtained showed occurrence of sensitive of the isolates to several antibiotics ,(Fig-1), ceftazidime (50%), cefixime (16.6%), ceftizoxime (75%), cefoxitin (33.3%), cefepime (91.6%), cephalothin (25%), sensitive to rifampin (83.3%), chloramphenicol (58.4%), trimethoprim/sulfamethoxazole (83.3%), streptomycin (91.6%), ofloxacin (100%), azithromycin (100%), kanamycin (75%), clarithromycin (83.3%), aztreonam (91.6%) piperacillin (83.3%), meropenem (100%) and sulbactam/ampicillin (33.3%), the isolates showed 100% resistance towards amoxicillin/clavulanic acid, penicillin and carbenicillin.
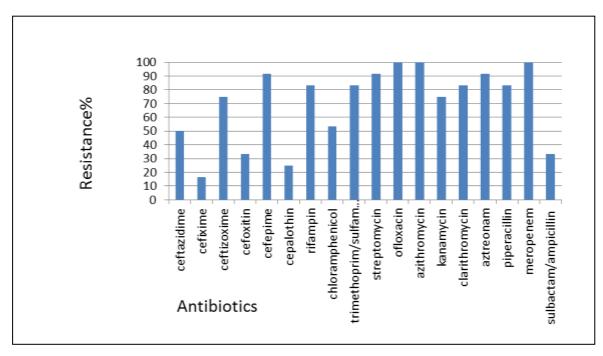

Fig.1 Antibiotic susceptibility of *Aeromonas hydrophila* isolated from patients with diarrhea.

MICs value for azithromycin range from 1-32 µg/ml ,ofloxacin range between 1-128 µg/ml , ,chloramphenicol was tested from 2-128 µg/ml and cefepime was the highest 512-1024 µg/ml (Table-3).

Table-3: Analysed by MICs, of 4 antimicrobial agents against 12 isolates of *Aeromonas hydrophila*

Antibiotic	No. of isolates at the following MICs(µg/ml)											
	0.5	1	2	4	8	16	32	64	128	256	512	1024
Azithromycin	0	5	1	3	0	2	1	0	0	0	0	0
Ofloxacin	0	3	2	0	3	0	2	1	1	0	0	0
Chloramphenicol	0	0	3	1	6	1	0	0	1	0	0	0
Cefepime	0	0	0	0	0	0	0	0	0	0	11	1

The haemolysin properties, protease ,phospholipase, DNase, slime production of *A.hydrophila* exposed to sub-MICs of azithromycin, ofloxacin, chloramphenicol and cefepime showed in Table(4).

Table-4: Virulence factors percentage under various values of sub-MICs

Virulence factors	sub-MICs of azithromy-cin	sub-MICs of ofloxacin	sub-MICs of chloramp-henicol	sub-MICs of cefepime
Haemolytic activity	33.3	-	41.6	16.6
Protease production	-	18.2	27.2	18.2
Phospholipase production	-	20	40	-
DNase production	-	-	44.4	22.2
Slime production	-	-	-	-

Discussion:

Diarrhea is one of the most common gastrointestinal illnesses, accounting for several billion cases annually throughout the world with 10 to 15 million deaths in the developing countries of Asia, Africa, and America [3]. In the current study 5.3% of patients was found to be infected with *A.hydrophila* that revealed an important association between diarrhea and *A.hydrophila*, our results is approximately agrees with study in south Brazil a bought the *Aeromonas* and associated with diarrheal diseases [17]. In recent year , *Aeromonas* spp. have been recognized with increasing frequency as a cause of gastrointestinal in children and adults, incidence of *Aeromonas* spp. causing a cute gastrointestinal ranges from(0.2%-7.7%) [18]. In this study, the isolation rate of *A.hydrophila* was obtained with a higher percentage in male than female, this observation is consistent with earlier study that demonstrated the isolation rate of *Aeromonas* spp. were higher in male than female patients [5]. In a study of the role of *Aeromonas* in diarrhea, member of the *A.hydrophila* group were isolated with equal frequency from both male and female patient [19]. The highest rates of *A.hydrophila* isolation was detected in children under 10 years of age, some studies showed that *Aeromonas* spp. were more prevalent in children aged less than 6 years[19], higher occurrence in adults aged 21-50 years was recorded [5]. These difference in results might be related to geographical locations, seasonal variation and identification techniques [3].

The occurrences of haemolytic factors in aeromonads are widespread [20]. 96% of *A.hydrophila* have haemolytic activity on human blood agar was reported [5] another study reported almost similar value of haemolytic activity equal to 95.2%, attention has been given on the haemolysin of motile *A.hydrophila* because the production haemolytic toxin has been regarded as

indication of pathogenic potential[9]. All *A.hydrophila* isolates with haemolysin positive genotype were virulent in the suckling mouse assay model [21]. Other authors reported the correlation between haemolysin and enterotoxin production, determined by suckling mouse test and they found that all entertoxigenic *A.hydrophila* isolates produced haemolysin [22]. Haemolytic activity is an index of pathogenicity and the relationship between the production of haemolysin and the enterotoxigenicity in *Aeromonas* spp. well documented [5].

Casein was easily digested by the *A.hydrophila* isolates [9] because the production of protease enzyme which detected in present study, other study showed that protease producers were 100% among the isolates, protease and haemolysin was found more frequently in the clinical isolates, which may be important in colonization through the disruption of the intestinal barrier [9]. The clinical isolates were phospholipase production, it is cytotoxic enzyme *A.hydrophila* reported the source of it, many effects belonged to this enzyme such as activity to digest the plasma membrane of host cell, damage to host cells leading to cell death and thought to be responsible for invasive properties [23]. Lipases were detected in 100% of clinical isolates of *A.hydrophila*, and it was associated to interaction with human leukocytes [17]. In the present study, we explored the DNase produce from diarrheal isolates of *A.hydrophila* which belong to the group of hydrolase [23]. DNase extracellular nucleases enzyme [13] was present more frequently in clinical than environmental strains [23], whereas, others refer that all *A.hydrophila* isolates produce DNase [24]. The study in Mexico of *Aeromonas* spp. Isolated from frozen fish, detected the DNase genes in 83% of the strains, although DNase activity was observed in all strains tested, this may indicate that there may be other DNase genes not targeted by primers that may be responsible for the activity characterization of *Aeromonas* spp. isolated from frozen fish [13]. Slime is another type of virulence factor, which is a viscous glycoconjugate material, produced by most of the Gram negative bacteria [20], which indicates the high-risk source contamination, they also found that 50 % of the clinical and 35.3 % of the environmental isolates were positive for slime production, the frequency of slime producing in clinical isolates was more [9]. In this study, we have shown that *A.hydrophila* isolates adhere to epithelial cells, the attachment to host cells is mediated by extracellular appendages of bacteria such as pili, and these filamentous structures were described as potential colonization factors in *A.hydrophila*, tow morphotypes of pili have been observed in *Aeromonas* spp. short rigid pili (s\r) type and long wavy flexible pili (l\w) type [23]. The clinical relevance of these adhesions is sometimes unclear, firstly because bacterial interaction with intestinal mucosa is complex, secondly, that epithelial cells were obtained from urine do not have the same surface receptors for bacterial adherence as those found on human intestinal cells in vivo, however, in several cases a correlation between adhesion in vitro and bacterial pathogenicity has been demonstrated [25]. The 12 *A.hydrophila* isolated in this study were tested for antibiotic susceptibility, conversely, all the isolates were resistant to penicillin, carbenicillin, amoxicillin/clavulanic acid, most of them were also resistant to sulbactam/ampicillin, furthermore, beyond the penicillins and first generation cephalosporins, some isolates showed resistance to several new extended-spectrum cephalosporins, and all the *A.hydrophila* isolates susceptible to azithromycine, some isolates could be considered as multi-resistant, as they stand to more than four antibiotics other than the β-lactam. In this sense, one isolate exhibited resistance to twelve antibiotics including (four penicillins, six cephalosporines, trimethoprim/sulfamethoxazole and kanamycine), tow isolates were resistant to (rifampine, cefixime, four pencillins), similar multiple antibiotic resistance patterns in *Aeromonas* were reported, indicating that this is a common and concerning feature of these bacteria, especially considering their ability to efficiently receive and transmit antibiotic resistance genes to other enteric species [17], the antibiotic therapy and drinking of untreated water are two significant risk factors for the susceptible host [26]. The results of the antibiotic sensitivity test indicate that ofloxacin, azithromycine, azteronam and meropenem might be effective agents for the treatment of diarrhea due to *A.hydrophila. A.hydrophila* was typically the most resistant species for *Aeromonas* species, antibiotic resistance mediated by plasmids seems to be of minor concern several chromosomally encoded, inducible β-lactamses have been

characterized it is speculated that resistance to multiple antibiotics in one *Aeromonas* isolate can be mediated by several conducible enzymes under the selection pressure of certain widely prescribed antibiotics [27]. In our study, distribution of MICs value of azithromycine and ofloxacin to *A.hydrophila* isolates were 1-32µg/ml, 1-128µg/ml respectively, they have a lowest MICs, 5 of 12 isolates being inhibited by a concentration 1µg of azithromycine per ml, 3of 12 isolates inhibited at 1µg /ml concentration of ofloxacin, these compound might become useful agents in the treatment of *A.hydrophila*, minimum inhibitory concentrations MICs of 5 resistance isolates of *A.hydrophila* to chloramphenicol ranged from 8-128 µg/ ml, whereas, the MICs of 7 sensitive isolates of *A.hydrophila* for the same antibiotic ranged from 2-8µg/ml. Chloramphenicol is hazardous to human, causing an idiosyncratic, aplastic anemia [6]. We showed that all isolates of *A.hydrophila* inhibited by 512µg/ ml of cefepime (fourth generation of cephalosporin) except one isolate inhibited at concentration 1024µg/ml. When exposure *A.hydrophila* to sub-MICs of selected antibiotics(azithromycine, ofloxacin ,chloramphenicol and cefepime), a decrement in the ability of haemolysin, protease, phospholipase, DNase and slime production was reported, these results are accordance with the findings of other authors who reported that concentrations of antibiotics lower than those necessary to inhibit growth (sub-MICs) can alter the ability of microorganisms to adhere to epithelial cells, change microbial toxin and enzyme production and increase microbial susceptibility to host defense mechanisms, and this may due to the action of azithromycin on the ribosome of bacteria which decreases protein synthesis and also the azithromycine binds to the 50S subunit [28]. For the ofloxacin it also showed an inhibition in the activity of haemolysin, protease, phospholipase, DNase and slime production, this might be due to the inhibition of DNA synthesis in bacteria which may cause several different metabolic and structural disorders [29]. Also an inhibition in the examined activities was recorded when chloramphenicol was used, this inhibition might be referred to inhibition in protein synthesis [29]. Another inhibition in the activities was noticed after the usage of cefepime this may be due to the stability against degradation enzyme and the ability to penetrate the porins in the outer membrane of gram negative bacteria this causes inhibition in cell wall synthesis [30]. .

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