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COMPARISON OF AEROKEYII SCHEME AND API20E SYSTEM FOR IDENTIFICATION OF *AEROMONAS HYDROPHILA* ISOLATED FROM WELLS WATER IN THI-QAR PROVINCE-IRAQ

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Key words : *A.hydrophila* , Wells, AerokeyII.

ABSTRACT

Untreated water is well-known source of *A.hydrophila* which is in addition to its enteropathogenic potential ,also display resistance to commonly used antibiotics , so this study aimed to compare Aerokey II and API20E in identification of *A.hydrophila* with studying of some virulence factors and antibiogram profile in untreated wells water in Thi-Qar province. Isolation was conducted by employing Ampicilin Blood Agar (ABA30) and MacConkey agar medium, the suspected colonies were identified by using biochemical scheme (AerokeyII) , and API20E . The results of this study revealed that *A.hydrophila* was recovered from 8 out of 30 wells with incidence rate (27.6%), incidence variation was noted among different regions which was statistically significant . All isolates showed β-hemolysis of human erythrocytes , 75% have proteolytic activity and 50% of isolates were DNase positive. Results of Antibiogram analysis revealed that all isolates exhibit resistance in percentage (100%) to five antibiotics including ;Clindamycin , Cephalothin , Vancomycin , ticarcilin-clavulanic acid , Ceftazidime, and the resistance to Cefoxitin was 75%, while all isolates were (100%) susceptible to Gentamicin , Amikacin, Chloramphenicol, Ofloxacin ,Ciprofloxacin, Naldxic acid, Imipenem , Norfloxacin, and Deoxycyclin. The susceptibility to Ceftriaxone was 62.5% , Streptomycin 87.5% and Trimethoprime 87.5% . The study concluded that , high correlation between Aerokey II and API20E in identification of *A.hydrophila* and untreated wells water are an important source of multi-drug resistant enteropathogenic *A.hydrophila* which pose public health threat especially to individuals using this kind of water source .

INTRODUCTION

Members of genus Aeromonas are roughly categorized into two groups based on their motility and growth temperature ; motile mesophilic and non-motile psychrophilic (1). Members of the mesophilic group have been implicated in intestinal and extra-intestinal infections ranging from acute gastroenteritis to life-threatening cases such as septicemia , necrotizing fasciitis and myonecrosis (2). Among the species belonging to this group , *A. hydrophila* has gained much interest due to its frequent association with human gastroenteritis ,which is occurring mainly in young, elderly and immunocompromised people (3).Untreated water is the most important source of Aeromonas infection , and these bacteria are commonly found in such water sources in developing countries(4). *A.hydrophila* has the ability to express a number of extracellular enzymes and toxins including protease; lipase, DNase, hemolysins and enterotoxins which have been considered as virulence factors (5). In early studies, it has been shown that enteropathogenic potential of *A.hydrophila* could be investigated in relation to their phenotypic markers including ; hemolysis of human red blood cells and Voges-Proskauer which are associated well with production of enterotoxins (6).

Multi-antibiotic resistant has been frequently identified in *A.hydrophila* more than other *Aromonas species* (7) .Much concern was paid for contribution of untreated water sources as path for dissemination of antibiotic resistance bacteria to humans and animals(8,9,10). Periodic monitoring of antibiogram profile of these bacteria in different geographical areas and from different sources is required for appropriate choice of antimicrobial agent for perfect therapy(11). According to our best

knowledge no previous study concerning the incidence and antibiogram profile of *A.hyrophila* isolated from well water in south of Iraq especially in Thi-Qar province , so this study aimed to isolate and identify *A.hydrophila* from well water in Thi-Qar province and to determine some virulence factors that are important in pathogenicity (phenotypically) with studying the antibiogram profile.

MATERIALS AND METHODS

Sampling

Ten milliliters (10 ml) of water sample was collected from thirty wells distributed in five regions (Shatrah, Nassiryah, Sukh-alshuikh, Said –Dukhail, and Batha) throughout Thi-Qar province .

Isolation and Identification of *A.hydrophila*

Water sample (10 ml) was aseptically added to 90 ml of Alkaline peptone water broth (APW) PH=8.6, and incubated at 37C ^{o} for 24 hours , then a loopful from growth film above APW broth was streaked on Ampicillin Blood Agar 30 and on MacConkey agar , all plates were incubated at 37C ^{o} for 24 hours. *A.hydrophila* identification was conducted based on biochemical scheme(AerokeyII) proposed by (12), and confirmed by API20E

Detection of virulence factors

The ability of isolates to hemolyze red blood cells was investigated on blood agar by observing hemolytic zone around colonies after 24 hours at 37C^{o} .

Protease activity was tested skimmed –milk agar plates . A loopful of an overnight growth from ABA30 was streaked on prepared skim milk agar plates , incubated at 37°C for 24 hours. The clear zone around colonies considered a positive result

DNase test was performed by inoculating bacterial culture on DNase medium (Oxiod) and incubated at 37C° for 24 hours . The positive result indicated by the presence of clear zone around the bacterial growth after adding HCL 0.1 N (13).

Antibiogram

Antibiotics sensitivity test was carried out according to Kirby-Bauer method using Mueller-Hinton agar . Following antibiotics discs were used , Gentamicin (10µg) , Amikacin (30µg), Streptomycin (10µg), Cholamphincol (30µg), Ofloxacin (5µg), Norfloxacin(10µg), Ciprofloxacin (5µg), Clindamycin(2µg), Nalidixic acid (30µg), Cephalothin (30µg), Cefoxitin (10µg), Ceftazidime (5µg), Ceftriaxone (30 µg),Doxycyclin (30µg),Vancomycin(30µg),Aztreonam(30µg), Trimethoprim-Sulfamethoxazole, Imipenem (10µg),and Ticarcillin-clavulanic acid (75/10 µg). The results of Antibiogram were interpreted based on criteria published in standards of the Clinical Laboratory Standard Institute Guidelines(CLSI,2011) (14) and the isolates were reported as susceptible, intermediate or resistant.

Multi-drug resistance index (MDRI) was calculated according to formula (15) :

$$MDRI = \frac{A}{B}$$

Where (A) the number of antibiotics to which the isolates showed resistance , (B) total antibiotics to which the isolates were exposed .

Multi-drugs resistance index (0.2) was considered as cut-off values in that value equal or higher than (0.2) was considered to have high risk source were antibiotic are often used , while value less than (0.2) indicated that strains are originated from source where antibiotics never used .

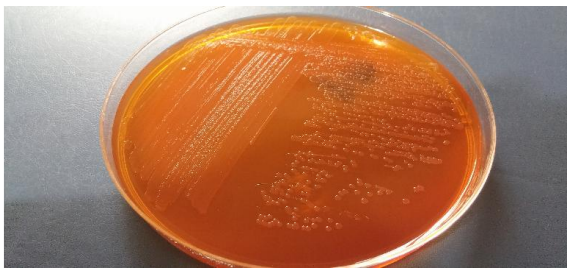
RESULTS

A.hydrophila recovered from 8 out of 30 (26.7%) wells selected from five regions that covering entire Thi-Qar province , incidence was differed among the five regions enrolled in this study, that the highest incidence 4(66.7%) was recorded in Batha region followed by 2(33.4%) in Sukh-Alshuikh and 1(16.7%) in each of Shatrah and Nassiryah ,while no *A.hydrophila* could be isolated from Said Dukail wells water table (1).

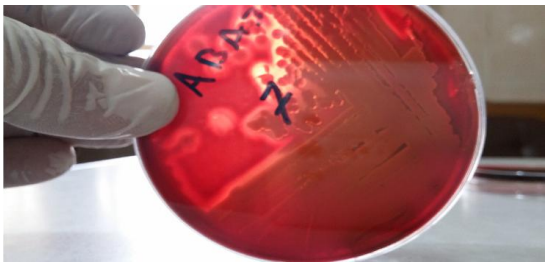
Table 1: prevalence of *A.hydrophila* isolated from wells water

Regions	No of samples	*A.hydrophila*	Percentage
Shatrah	**6**	**1**	**16.7%**
Nassiryah	**6**	**1**	**16.7%**
Sukh-alshuikh	**6**	**2**	**33.4%**
Said –Dukhail	**6**	**0**	**0%**
Batha	**6**	**4**	**66.7%**
Total	**30**	**8**	**26.7**
X^2 *P-Value*	**12.589 (p<0.05)**		

All isolates were able to grow on ABA30 with smooth white to buff color surrounded with β-hemolysis after 24 hours of incubation at 37C°, indicated that all isolates were resistance to ampicillin and able to hemolysis human red blood cells .On MacConkey agar plates, all isolates produce large colorless colonies indicated that all isolates were unable to ferment lactose figures (1 and 2).



Figure(1): Colorless colonies of *A.hydrophila* on MacConkey agar plate



Figure(2):Growth of *A.hydrophila* on ABA30, with β-hemolysis

The results of AerokeyII biochemical scheme were presented in table (2). All isolates were positive for Indole , Citrate utilization , and fermentation of glucose and oxidase , while all isolates were negative for string test and resist O/129 which are the key tests for differentiating *Aeromonas* from *Vibrio* . All isolates were , esculin hydrolysis ,

produced acid from arabinose and resistant to Cephalothin, motile (hanging drop) , and majority of isolates were Voges-Proskauer positive.

Table (2) Results of biochemical tests for identification of *A. hydrophila*

Test	Reaction	No postive (%)
Growth on ABA30	+ β-hemolysis	8/8 (100%)
Indol	+	8/8 (100%)
Citrate utilization	+	8/8(100%)
Glucose fermentation with gas	+ Gas production	8/8(100%)
Oxidase	+	8/8(100%)
String	-	8/8(100%)
Resistance to O/129	+ Resistance	8/8(100%)
Motility	+	8/8(100%)
Esculin hydrolysis	+	8/8(100)
Voges-Proskauer	+	6/8 (75%)
Acid from arabinos	+	8/8(100%)
Resistance to Cephalothin $_{30\mu g}$	+ Resistant	8/8(100%)

A.hydrophila were also subjected to API20E (analytical profile index , bioMérieux) and results of current study indicated that biochemical tests in Aerokey II are highly discriminative and are excellent in identification of *A.hydrophila* to species level as all positive isolates were also identified by API20E as *A.hydrophila*

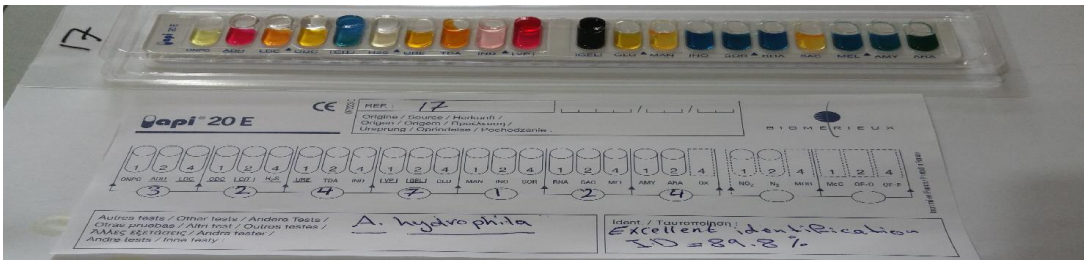


Figure (3) shown API20E (analytical profile index) result of *A.hydrophila* identification according of API20E data

Detection of virulence factors revealed that all isolates in the current study were

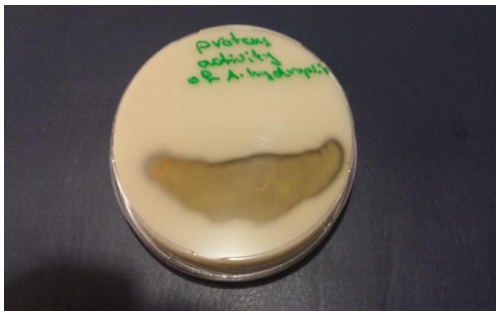
Virulence factor / Region	No of isolates	Protease	DNase	Hemolysis
Shatrah	1	1(100%)	1(100%)	1(100%)
Nassiryah	1	1(100%)	1(100%)	1(100%)
Sukh-alshuikh	2	1(50%)	0(0%)	2(100%)
Said –Dukhail	0	---	----	----
Batha	4	3(75%)	2(50%)	4(100%)
Total	8	6(75%)	4(50%)	8(100%)

Table (3): Virulence factors of *A.hydrophila* from wells water in different regions

able to express virulence factors , that all isolates were β-hemolytic on blood agar regardless of region of isolation , and 75% of these isolates were positive for protease test and 50% were positive in DNase test ,figures (4 and 5). *A.hydrophila* isolates from Shatrah and Nassiryah wells water were expressing the three virulence factors ,while the isolates from Sukh-alshuikh showed no DNase activity and only one isolate was expressing protease activity , while three isolates from Batha were protease positive and two isolates were DNase positive , table (3).

Figure (4): DNase activity of *A. hydrophila*

Figure (5) protease activity of *A.hydrophila* on Skimed-milk agar



and Trimethoprim-Sulfamethoxazol have excellent activity (≥ 80% of isolates were susceptible) while Certiaxon had moderate susceptibility (60% of isolates were susceptible) and low susceptablitiy (≤30% of isolates were susceptible) was recorded for Cefoxitin .

Table (5): Antibiogram Profile of *A.hydrophila* Isolated From Wells Water

Table (4): Antibiogram profile of *A.hydrophila*

Antibiotics	Potency	1	2	3	4	5	6	7	8	Diameter of zone			Susceptibility (%)
										R	I	S	
Gentamycin	10	S	S	S	S	S	S	S	S	12	13-14	15	100%
Cholamphincol	30	S	S	S	S	S	S	S	S	12	13-17	18	100%
Ofloxacin	5	S	S	S	S	S	S	S	S	12	13-15	16	100%
Amikacin	30	S	S	S	S	S	S	S	S	14	15-16	17	100%
Clindamycin	2	R	R	R	R	R	R	R	R	14	15-20	21	0%
Naldxic acid	30	S	S	S	S	S	S	S	S	13	14-17	18	100%
Streptomycin	10	S	S	I	S	S	S	S	S	12	13-16	17	87.5%
Cephalothin	30	R	R	R	R	R	R	R	R	14	15-17	18	0%
Norfloxacin	10	S	S	S	S	S	S	S	S	14	15-18	19	100%
Vancomycin	30	R	R	R	R	R	R	R	R	-	-	17	0%
Doxycyclin	30	S	S	S	S	S	S	S	S	10	11-13	14	100%
Aztreonam	30	I	I	I	I	I	R	I	I	15	16-21	22	--
Cefteriaxon	30	S	I	S	I	S	S	S	I	13	14-20	21	62.5%
Trimethoprim-sulfamethaxazon		S	I	S	S	S	S	S	S	10	11-15	16	87.5%
Imipenem	10	S	S	S	S	S	S	S	S	13	14-15	16	100%
Ticacillin-clavulanic	75/10	R	R	R	R	R	R	R	R	14	25-19	20	0%
Ciprofloxacin	5	S	S	S	S	S	S	S	S	15	16-20	21	100%
Ceftazidime	5	R	R	R	R	R	R	R	R	14	15-17	18	0%
Cefoxitin	10	R	S	R	R	R	S	R	S	14	15-17	18	25%
MDRI		0.31	0.26	0.31	0,31	0.31	0.31	0.31	0.26				

On the other hand absolute resistance (100%) of *A.hydrophila* was observed against five antibiotics : Ticacillin-clavulnic , Ceftazidime, Vancomycin, Cephalothin, and Clindamycin, while the resistance to Cefoxitin was 75% . In this study two isolates were (25%) resistant to at least five antibiotics with multi-drug resistance index MDRI(0.26) and six isolates were (75%) resistant to six antibiotics with MDRI(0.31).

Discussion

Exposure of individuals to water contaminated with *Aeromomas sp* has been reported to be an important cause for human diarrheal disease , as these bacteria able to produce enterotoxins , cytotoxins, and hemolysins (2) .

The results of this study in concerning incidence of *A.hydrophila* in wells water was (26.7%) convergent to those reported by previous studies including ; (59%) in Libya reported by (16) and (48%) reported by (17) and 50% reported by (18) in Iraq .

The low incidence rate of *A.hydrophila* reported in current study could be attributed to isolation procedure as they used lower Ampicillin concentration 15mg/L incorporated in blood agar (30 mg/L in recent study) ,or due to limited number of wells enrolled in this study or due to investigation the prevalence of genus Aeromonas without performing species identification (17) however, the results of this study were in concordance with the results obtained by (19) and (20) in brazil as they found *A.hydrophila* incidence in well water was 22.5% and 21.9% respectively, and agreed with (21) who found the incidence of *A.hydrophila* in well water (27%) and 28% reported by (22) . In this study there was variation in prevalence of *A.hydrophila* from wells water among five regions enrolled. variation in recovery of Aeromonas from different geographical locations was documented by (23).

Identification of Aeromonas to species level based on phenotype is necessary since there are differences in clinical importance , antibiotics susceptibility and epidemiology of different species of Aeromonas (24) . In this study *A.hydrophila* has been identified to species level by using a set of highly discriminatory biochemical scheme (AerokeyII), that have been proposed by (12),by this scheme he was able to identify 97% of Aeromonas to species level and 100% of reference strains . The

results of this study found that Aerokey II is an excellent method for identification of *A.hydrophila* , this result was compatible with, (25) and (24) who found that API system alone cannot identify Aeromonas to species level unless this system used in combination with Aerokey II .

Some researchers have stated that the hemolytic activity , or hemolytic activity with voges-Proskauer positive , act as a good indicator of ability of these bacteria to produce enterotoxins (6,26) . According to this criteria , majority of isolates in this study were enterotoxigenic , as 100% of isolates were β-hemolytic and 75% of isolates were V-P positive , this result was agreed with (26,27,28) .

In this study all isolates (100%) were multi-drug resistance with MDRI ranging from 0.26-0.31 , Multi-drug resistance phenotype among *Aeromonas spp* has been reported from different regions throughout the world , particularly in *A.hydrophila* (10). The results of this study were agreed with (29,30,31). The resistance of all isolates to Ticarcillin/clavulanic acid and cephalothin was not unexpected because *A.hydrophila* classically resistant to Beta –lactam antibiotics, this resistance can be explained in a part that *Aeromonas spp*. isolates are resistant to many β-lactams as a result of multiple inducible, chromosomally-encoded β-lactamases which are under a single mechanism of coordinate expression (31), and among *Aeromonas spp*, only *A.hydrophila* express four classes of -lactamases A;B ,C, and D class(**32**) , the resistance of *A.hydrophila* to many β-lactams antibiotics including ampicillin is a significant public health concern , as this property may act as predisposing factor for gastroenteritis , since ampicillin inhibiting competing microflora in intestine, that is benefit for *A.hydrophila* to proliferate and producing toxins (33).

Our study indicated Aminoglycosides (Gentamicin, Amikacin) ,Quinolone and Flouroquinolones, Carbapenem, Doxycyclin and Chloramphenicol were absolutely

active against *A.hydrophila*, this finding was in agreement with previous studies (11,29, 30, 31). In this study we have isolated *A.hydrophila* from wells water , that have the ability to express virulence factors and majority of these isolates were enteropathogenic and expressed resistance to most commonly used antibiotics including the third generation of cephalosporins , this may constitute an important health risk to population that used untreated water sources for daily usage .

مقارنة المخطط التشخيصي (AerokeyII) و أشرطة الفحص السريع (API20E) في تشخيص الايروموناس هايدروفيلا المعزولة من مياه الآبار في محافظة ذي قار – العراق

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الخلاصة

تعتبر المياه الغير معاملة مصدر مهم لبكتريا الايروموناس و التي تعتبر بكتريا معوية ،ولها القدرة على مقاومة العديد من المضادات الحيوية شائعة الاستخدام ، لذا هدفت هذه الدراسة إلى مقارنة المخطط التشخيصي (Aerokey II) و أشرطة الفحص السريع (API20E) في تشخيص هذه البكتريا و دراسة بعض عوامل الضراوة و نمط مقاومة المضادات الحيوية لهذه البكتريا في مياه الآبار في محافظة ذي قار . استخدم وسط اكار الدم المزود بالامبسلين بتركيز 30 ملغرام/لتر و وسط المكونكي لعزل البكتريا و استخدم المخطط التشخيصي(ArokeyII) و أشرطة الفحص السريع (API20E)في التشخيص . وجدت الايروموناس هايردوفيلا في 8 أبار من أصل 30 بئر بواقع (27.6%) كذلك أظهرت هذه الدراسة اختلاف في نسبة العزل بين المناطق و بفرق معنوي عند مستوى المعنوية (0.05). و فيما يخص عوامل الضراوة فقد بينت هذه الدراسة أن كل عزلات الايروموناس هايروفيلا (100%) كانت قادرة على تحليل الدم باستخدام وسط اكار الدم و 75% منتجة لانزيم البروتيز و 50% قادرة على انتاج انزيم DNase . اظهر فحص المقاومة للمضادات الحيوية أن كل العزلات (100%) كانت مقاومة لخمسة انواع من المضادات الحياتية وهي : , Clindamycin Cephalothin , Vancomycin , ticarcilin-clavulanic acid , Ceftazidime وكانت نسبة المقاومة

لل Cefoxitin 75% . بينما كانت جميع العزلات(100%) حساسة للمضادات الحياتية , Gentamicin Amikacin, Chloramphenicol, Ofloxacin , Ciprofloxacin, Naldxic acid, Imipenem , Norfloxacin, and Deoxycyclin و كانت الحساسية للCeftriaxone (62.5%) و Streptomycin 87.5%و Trimethoprime 87.5% . من خلال نتائج هذه الدراسة نلاحظ وجود توافق بين التشخيص بكلا الطريقتين و كذلك تعتبر الآبار مصدر مهم لبكتريا الايروموناس هايدروفيلا التي لها القدرة على إظهار مقاومة متعددة للمضادات الحياتية و القادرة على أنتاج السموم المعوية و التي تشكل خطر على الصحة العامة خصوصا بالنسبة للأشخاص الذين يعتمدون على هذا النوع من مصادر المياه .

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