Antibiotics Susceptibility of Bacteria Aeromonas Hydrophila

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Abstract

From a group of (294) children aged (6) years old from the second half of 2012, a sample of stools was collected from children aged from one month to 2 years with diarrhea from Iraq.

The samples were subjected to the laboratory methods and PCR-SSP technique in the city of Samaa in the years 2013-2014.

Aeromonas hydrophila was isolated from 12 stool samples. The results showed that all isolates were able to produce hemolysin and protease and lipase and phospholipase and also contained their enzymes.

Also, these isolates were resistant to some antibiotics such as amoxicillin and ampicillin and ceftriaxone and cefotaxime, whereas all isolates were sensitive to naldixic acid and tetracycline and gentamicin.

Keywords: Diagnosis of some virulence factors and the effect of some antibiotics. Aeromonas hydrophila
1. Introduction:

Aeromonads are microorganisms living water, and is often found in chlorinated and non-chlorinated water and bottled drinking water supplies in bottles. As it has been isolated from food. Therefore, food and water from potential sources of human infection [1]. Some species of aeromonads cause opportunistic infections in humans and diseases of aquatic animals [2]. Aeromonas is mostly known as an enteric pathogen. A strong correlation between gastroenteritis and Aeromonas species has been shown in children, adults who are older than (60) years and in cases of traveler’s diarrhea [3]. In fact, the three most common human infections caused by Aeromonas species are gastrointestinal infection, skin and soft-tissue infection, and bacteremia in immunocompromised individuals[4]. Aeromonas hydrophila are most often the advantage of being caused intestinal and extraintestinal human infections [1]. The potential virulence of aeromonad that lead to the pathogenicity include, cell structural (cell-associated structures) outer-membrane proteins, lipopolysaccharides, flagella and pili, a type III secretion system (T3SS) acting as adhesion structures, and extracellular factors such as enzymes and toxins [5,6]. A. hydrophila virulence factors that cause diarrhea which include intestinal cytotoxic and intestinal cytotoxic [7,8,9]. As well as multiple drug resistance occurred more in A. hydrophila than other species of Aeromonas spp and that isolates from humans and animals are more resistant to antibiotics [10].

2. Materials and Methods

2.1. Samples Collection and Identification of Aeromonas hydrophila

A total of (294) diarrheic stool samples were collected between December (2012) and February (2013) from Al-Muthana public health laboratory in Muthana province - Iraq. All suspected isolates were screening by traditionally tests and then confirmed by PCR technique (16S r RNA gene). The results showed that; there were (12) positive isolates of Aeromonas hydrophila. Then investigation was done on some virulence factors and all isolates were showed its ability to produce haemolysin, protease, lipase, phospholipase, capsule and motility. As well as, the antibiotic susceptibility of these isolates were taking in the account and showed that all the isolates were multiple antibiotic resistance to the Amoxicillin, Ampicillin, Cefalothin and Cefotaxime. While, all isolates were sensitive to the Nalidixic acid, Tetracycline and Gentamicin.

Abstract

A total of (294) stool samples were collected from patient children, their ages were between 1 month to (6) years and suffering from diarrhea disease during period December (2012) and February (2013) from Al-Muthana public health laboratory in Muthana province - Iraq. All suspected isolates were screening by traditionally tests and then confirmed by PCR technique (16S r RNA gene). The results showed that; there were (12) positive isolates of Aeromonas hydrophila. Then investigation was done on some virulence factors and all isolates were showed its ability to produce haemolysin, protease, lipase, phospholipase, capsule and motility. As well as, the antibiotic susceptibility of these isolates were taking in the account and showed that all the isolates were multiple antibiotic resistance to the Amoxicillin, Ampicillin, Cefalothin and Cefotaxime. While, all isolates were sensitive to the Nalidixic acid, Tetracycline and Gentamicin.

Keywords

A. hydrophila isolation, investigate of some virulence factors and antibiotic susceptibility.

<table>
<thead>
<tr>
<th>Primer type</th>
<th>Primer sequence</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward 16Sr RN4 - F</td>
<td>5-CCAGCAGCGC GGTAATACG-3</td>
<td>300 bp</td>
</tr>
<tr>
<td>Reverse 16Sr RN4 - R</td>
<td>5-TACCAGGTATCTAATCC-3</td>
<td></td>
</tr>
</tbody>
</table>

Table (1): The sequence of forward and reverse primers
According to information of manufacturing company (Master mix, Geneaid/Taiwan) PCR mixture solution and PCR Program conditions was listed in Table (2) [11]. In first well on (1%) agarose gel ten ml standard molecular weight of DNA ladder (marker) was loaded and each well has been loaded with (10) ml of PCR product (DNA sample). Electrophoresis runs at (80) volt/cm for 1hr.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
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</tr>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
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<td>30 sec</td>
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</tr>
<tr>
<td>Elongation</td>
<td>72 °C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Final elongation</td>
<td>72 °C</td>
<td>10 min</td>
<td></td>
</tr>
</tbody>
</table>

2.2. Investigate of Some Virulence Factors

2.2.1. Protease activity assay

Protease medium was prepared by the following solutions:-
1. Add 10g from skim milk to (90) ml of D.W and completed to the (100) ml, then, gently heated at (50)°C, and without autoclave.
2. Add (2)g of agar powder to the (100) ml of D.W and mixed thoroughly, then autoclaved, and cooled to (50-55)°C.
3. Preparation medium:- (100) ml of solution (1) has been mixed with (100) ml of solution (2) directly. Then was poured into sterile Petri dishes and by streaking method a single colony was cultured on skin milk agar and was incubated at (37) °C for (24- 72) hr at (37)°C. As a positive result the color of precipitation zone around the colonies should change from white to brown color [16].

2.2.2. Phospholipase activity assay

Phospholipase medium was prepared by dissolving (1g) of NaCl in (100) ml of nutrient agar. Then sterile by autoclave and was cooled to the (50)°C. After that in sterilized conditions one egg yolk has been added to the mixed medium and mixed well. Then, the mixed medium has been poured into the sterilized Petri dishes and inoculated with a single colony and incubated for (24-72) hr at (37)°C. A spread growth out of stab line indicate positive result appeared when the was [12].

2.2.3. 1%Tween20 media of lipase activity assay

This media was prepared according to the [17] as follows:- (1) ml of (1%) Tween 20 was added to (100) ml of nutrient agar after that sterilized by autoclaving and poured in to sterilized Petri dishes and inoculated with a single colony and incubated for (1-5) days at (37)°C. After that the motility medium has been distributed in tubes then used to detect bacterial motility. Then the tubes that were contained semisolid media inoculating with bacteria by a stabbing method and has been incubated at (37)°C for (24-48) hr. A spread growth out of stab line indicates positive result.

2.2.4. Capsule test

A colony of young bacterial isolates was mixed with drop of normal saline on clean slide and late smear to dry at room temperature (don’t use flame). Then crystal violate stain was added to (2-3) min, after that discard the stain and wash the slide by (20%) CuSO4.7 H2O (don’t use water) and examined under microscope. Capsule appears as a clear zone around the body of the bacteria which appeared blue [18].

2.2.5. Motility

To prepare motility medium (4) gm of agar added to (100) ml of nutrient broth after that completed up to (1000) ml with D. W. Then it has been sterlized by autoclave at (121) °C for (15) minutes. After that the motility medium has been distribut-ed in tubes then used to detect bacterial motility. Then the tubes that were contained semisolid media inoculating with bacteria by a stabbing method and has been incubated at (37)°C for (24-48) hr. A spread growth out of stab line indicate positive result appeared when the was [12].

2.3. Antimicrobial Susceptibility

According to [19] the antibiotics and its standard inhibition diameters was used to detect antimicrobial susceptibility. To prepare the inoculums (3-5) isolated colonies grown on nutrient agar plate has been added to (5) ml of sterile normal saline and then compared with (1.5X 108) cf/ml McFarland standard tube. To obtain inoculums the multiple antibiotic resistance index (MAR) was calculated for each isolate separately and determined by divided the number of antibiotics, on which the isolate is resistant over the total number of antibiotics, towards which the isolate susceptibility was checked. The MAR index, that was higher than 0.2 (>0.2) identifies bacteria isolated from objects with higher risk of contamination, whereas antibiotics was often used. The MAR index (≤ 0.2) identifies strains from the environment, however, antibiotics are not used at all or rarely used [20].

\[
\text{MAR index} = \frac{\text{Number of antibiotics which the isolate is resistant}}{\text{Number of antibiotics which the isolate susceptibility has been checked}}
\]

3. Results and Discussion

3.1. Samples Collection and Identification of Aeromonas hydrophila

In this study (12) isolate obtained from (294) diarrheic stool samples that were collected from Al-Muthana public health laboratory. To confirm initial diagnosis of bacteria a manual bio-chemical tests were used Table (3) have shown that A.hydrophila was presented a positive result for each of oxidase, catalase, methyl red, gelatin liquefaction , simmone citrate, and Indole. The finding results of this study are almost similar to other researchers reports [21,22]. Moreover, A.hydrophila has the ability to ferment glucose on from the bacterial suspension A sterile swab has been used in this technique . After that the inoculums has been streaked on a Mueller-Hinton agar (MHA) plate then left to dry. The antibiotic discs has been placed on the surface of the medium at equally spaced intervals with flamed forceps or a disc applicator then it was incubated for (24) hours at (37)°C. To determine the sensitivity or re-sistance of the organism to each antibiotic the inhibition zones were measured and compared with the zones of inhibition determined by the Clinical Laboratory Standards Institute [12].
in terms of positive results, while, it is agree with [12,23] in the negative results. All of A. hydrophila isolates gave negative results to string test (sodium deoxycholate). This test considered to be a differential test between A. hydrophila and V. cholerae [21]. In addition PCR technique was used to confirm identify A. hydrophila and all isolates have given a positive result for 16S rRNA gene [11] Fig (1).

3.2. Virulence factors

Number of virulence factors of A. hydrophila isolated from patients children whom suffering from diarrhea disease were detected. The results revealed that all A. hydrophila isolates were positive to the β-haemolysin. The results of this study in agreements with [5,2] whom described that; β-hemolysins as an important bacterial virulence factors which promoting channel formation leading to cell death. As well as, all the isolates showed positive results to phospholipase test. The phospholipase produce a precipitate zone when grown on egg yolk medium according to [24]. This is agreed with [25,26] whom indicated that phospholipases play an important role in pathogenesis of A. hydrophila. Furthermore, all isolates gave a positive results to protease test when was cultured on skim milk media. Another study noticed that A. hydrophila has the ability to produce protease enzyme that act to hydrolyze protein [27]. Additionally, A. hydrophila isolates presented positive results to lipase test that able to hydrolyze fat. Also [28] indicated that A. hydrophila is known to secrete lipases and have been suggested that lipases may supply nutrients and contribute virulence factors by react with human leukocytes or by affecting many immune system functions through free fatty acids generated by lipolytic activity [29].

In addition, all isolates showed positive results to the biofilm formation. In their reports [30,31] presented that the persistence of A. hydrophila in biofilms within water distribution systems, as well as, their multiple resistance (R), after isolated from these utilities were for a long time undervalued, while these aspects are being important for the public health [32,33]. Moreover, all isolates of A. hydrophila exhibited capsule. The results of this study are also agreed with the finding of [33] which is the role of A. hydrophila capsule polysaccharide as virulence factor since most motile strains. Likewise, A. hydrophila isolates appeared a positive result to motility after inoculated on semisolid media and that leads to disseminating of growth out of the stab line after indication. This result agreed with [4] who showed that A. hydrophila composed of motile isolates which grew well at (35 to 37)°C and was associated with a variety of human infections. Table (4).

3.3. Antimicrobial Susceptibility

The antibiotics sensitivity of A. hydrophila isolates were investigated according to [19,34]. The results that obtained from this study(Fig 2) revealed that all A. hydrophila isolates were gave 100% resistant to the Amoxicillin, Ampicillin, Cephalothin and Cefotaxime, while A. hydrophila isolates were showed resistance rates 91.66%, 66.66%, 58.33%, 50%, 41.66, 33.33%, 25% and 8.33% to Imipenem, ceftriaxone, Ciprofloxacin, Trimethoprim, Tobramycin, Amikacin, Chloramphenicol and Norfloxacin respectively.
However, all A. hydrophila isolates were totally sensitive 100% to the Nalidixic acid, Tetracycline and Gentamycin. The obtained results of this study is almost in agreements with [35] in terms of resistance rates for Cephalothin ,Amoxicillin/sublactam, Ampicillin, Cefotaxime, Imipenem, ceftriaxone, Trimethoprim/sulfamethoxazole, Tobramycin and Chloramphenicol, and also in terms of its sensitivity to Nalidixic acid. So the finding of this study agreed with the results that obtained from both [35,36] as A. hydrophila was isolated from patients children whom suffering from diarrhea. While the results disagreed with [35] results in terms of A. hydrophila susceptibility to Tetracycline, Gentamycin, Ciprofloxacin, Amikacin and Norfloxacin.

On the other hand, there are similarities in the results that obtained from the current study with [11] finding in terms of A. hydrophila isolates susceptibility to Amoxicillin, Ampicillin, Cephalothin, Gentamycin and Nalidixic acid, but it was differ in its susceptibility to Tetracycline, Chloramphenicol, Tobramycin, Ciprofloxacin, Trimethoprim and Ceftriaxone.

In fact, the key word behind the differences in A. hydrophila resistance to antibiotic is that most of aeromonads produce an inducible chromosomal β – lactamase with activity against a wide variety of β-lactam antibiotics [37], moreover, antibiotics resistance to chloramphenicol, streptomycin, tetracycline, cefoxitin, cephalixin, erythromycin, furazolidone, and sulfathiazole is mediated by plasmids [38], [39] observed that the treatment of bacterial-associated diarrhea become more intricate by the frequency of antimicrobial resistance in many pathogens. Data concerning antimicrobial resistance for A. hydrophila have been varied.

3.4. The index of multiple antibiotic resistances :-
In this study the index of multiple antibiotic resistances (MAR) has been calculated for A. hydrophila isolates and result was 0.26 for all isolates, and was indicative to wide antibiotics usage. As it was presented by [40,41] reports whom determined MAR for a number of A. hydrophila strains.

![Antibiotics susceptibility of A. hydrophila isolates.](image)

**Antibiotics**

- AX: Amoxicillin
- IPM: Imipenem
- TOB: Tobramycin
- AK: Amikacin
- KP: Cephalothin
- CP: Ciprofloxacin
- CTS: Cefotaxime
- TMP: Trimethoprim
- NX: Norfloxacin
- NA: Nalidixic acid
- TE: Tetracycline

![Fig. (2): Antibiotics susceptibility of A. hydrophila isolates.](image)

**References**


