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Detection of *Aeromonas* spp. by traditional and molecular methods from tap water in Baghdad city

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Abstract:

Water borne diseases are caused by pathogenic microorganism's viruses, bacteria, intestinal parasites and other harmful microorganisms, which are directly transmitted when contaminated fresh water is consumed.Most of enteritis cases caused by drinking contaminated water with pathogenic bacteria such as *Aeromonas* spp, determination of its virulence factors very important especially its ability to produce toxins.One thousand five hundred sixty seven water samples from different parts of Baghdad city were collected from the beginning of April 2010 till the end of December 2011, all samples were bacteriologically examined by traditional methods for detection of Total coliform and other pathogenic bacteria. Thirteen isolate of *Aeromonas*spp were isolated and tested for its pathogenicity and ability to toxin production in the mouse module, all environmental isolates induced fluid accumulation (FA ratio≥100) after 5 hours and cause histopathological effects after 24h of inoculation. Histopathological changes showed severe inflammation of the mucosa and submucosa in the small intestine with mild chronic inflammatory cells and shortanage of villi, mild degenerative of gastric mucosal glandular epithelial and slightly necrosis, massive necrosis of hepatic cells with infiltrate of chronic inflammatory cells in the liver section،while no effect appeared on kidney tissue. All isolated of *A. hydrophila* were analyzed for conventional PCR for detection of virulence factors using *Fla* and *laf* flagell in genes most of isolates gave positive results for boththese genes.

Introduction

Water we drink has essential ingredients for our well being and a healthy life, unfortunately polluted water and air are common throughout the world. The WHO states that one sixth of the world's population, approximately 1.1 billion people do not have access to safe water and 2.4 billion lack basic sanitation [1]. Polluted water consists of industrial discharged

effluents, sewage water, rain water pollution and polluted by agriculture or households cause damage to human health or the environment [2]. This water pollution affects the health and quality of soils and vegetation. Some water pollution effects are recognized immediately, where as others don't appear up for months or years [3].

In Iraq water contamination still pose a big health problem, many pathogens have been isolated mainly *Salmonella* spp.,*V.cholera* , studies showed that this problem peaked in summer more than other seasons [4], [5]. Several methods have been used for isolation and detection of pathogenic bacteria and *Aeromonas spp.* from water samples, such as traditional and molecular methods. Polymerase chain reaction, (PCR) technique has been conducted successfully to detected small amounts of DNA pathogens in aquatic environments with high sensitivity and specificity.

The major aims of this study was to develop a rapid method for the observing of *Aeromonas spp.*in drinking water, through detection of some virulence genes such as *Laf*, *Fla* genes [6], [7].

Material and Methods

1-Water sample collection:-

One thousand five hundred sixty seven of tap water samples were taken from different parts in Baghdad area, during the period 1st of April 2010 till 31 of December 2011. One litter of each sample was collected in a sterile bottles with screw cap, containing sodium thiosulphate 0.1% to neutralize the residual chlorine, and transported in an cooling aspectic container to the laboratory within 2-6 h forimmediate bacteriological analysis and /or stored at 4°C for up to24h to use, for further investigation.

2-Bacteriological Examination: -were carried out according to [18]

Traditional methods:-

1- Isolation of of Total coli form(TC) , fecal coli form (FC), and *Escherichia coli* through using the membrane filter technique (MF), tube fermentation test

2- (TFT) and presence /absence/ (P/A) method according to [7].

Isolation and Identification of *Aeromonas* spp.:-

1- Water samples were passed through 0.45 µm Millipore filter paper, which was transferred to the alkaline peptone water, incubated at 35ºC for 24 h, streaked on ampicillin dextrin agar (ADA) for isolation of *Aeromonas* spp. incubated at 35 ºC for 24 h.

2-Yellow bright colonies 1-1.5 mm in diameter, well appeared, 2-3 colonies were picked and re-inoculated on nutrient agar at 37ºC for 24h.

3-Colonies for oxidase production were tested, positive colonies were further tested for production of hemolysine.

4-Colonies picked and inoculated onto sheep blood agar plates, incubated at 35ºC for 24h for hemolysine production.

3-Molecular Identification of *Aeromonas ssp.:-*

DNA extraction: - Two method were used for extraction:-

- Extraction of DNA from isolated bacteria,carried out by using genomic DNA kit (Gene aid).

- Total genomic DNA was isolated directly from water samples according to [7] with few modifications as follow:

1- Water samples were concentrated by filtration through 0.45-µm-pore size nitrocellulose filters.

2- The filters were then vortexes in peptone broth for recovering bacteria and then incubated at 37°C for 24 h.

3- Suitable volume (1.5ml) of growth liquid wascentrifuged at 4500 g for 20 minute. The pellet was extracted with protocol of genomic DNA extraction kit.

4- PreservedDNAwith 50-100µl of TE solution in Eppendorf tubes at 20- Cº.

4-Detection of virulence factors in *Aeromonas ssp.*:- which was performed by

1-Animal model: - The enteropathogenicity of *Aeromonas*spp. was carried out as described by [8] using the sealed-adult-mouse model Swiss albino mice weighing about 15–20 g. The animals, were kept in sterilized cages with autoclaved bedding, were acclimated to laboratory conditions (12 h dark: 12 h light cycles; 24±1 °C). The procedure was carried out as following:-

1- The isolated strains were grown in brain heart infusion brothat 37°C with shaking for 24h, harvested by centrifugation.

2- Eighteen mice were divided into three equal groups ,the first group regarded as control, the second and third were consider as treated groups which were killed,depending on the time 5h ,24 hfor detection the effects of the toxins on organs.

3- After 15 min, the bacterial inoculate (1×1^{10} CFU/ml) in 200 µl of PBS were given orally to the test animal.

4- At 5 h from post-inoculation, the animals of secondgroup were sacrificed, and the fluid accumulation (FA) ratios were determined, FA ratios of ≥100 were considered positive.

5- For the colonization assay, infections were allowed to proceed for 24 h. The mice of third group were sacrificed, and the intestines, stomach, kidney, and liver were removed and kept for histopathological examination.

Histopathological examination: -Histopathology was performed as described by [9]. After 24 h post-inoculation, mice were euthanized and sections of small intestine, stomach, kidney andliverwere immediately fixed in 10% neutral buffer formalin. Following fixation, tissue samples were embedded in paraffin, sectioned at 5 µm and stained with haematoxylin-eosin for light microscopic examination.

2-Molecular methods:-

PCR used for detection of the *Laf, Fla* genes for conformation the identification of the *Aeromonas spp*., according to [6] and [Santons*etal.* (2010]. these primers synthesized by Cinna gen company (Table 1).

Table (1): The sequence and concentration of forward and reverse primers of*Laf*and*Fla* genes.

Primer type	Primer size	Concentration in bicomole	Product size
Laf F	-GGTCTGCGCATCAACTC-	37881.12	504bp

Laf R	-GCTCCAGACGGTTGCTG	23592.32	504bp
Fla F	-TCCAACCGTYTGACCTC	38282.63	608bp
Fla R	-GMYTGGTTGCGRATGGT	33707.87	608bp

PCR reaction was conducted in 100µl of reaction mixture containing 50 µl of green master mix,5 µl of each primer,10 µl DNA template and 30 µl of deionized water (Table 3-12).

Table (2): The mixture of conventional PCR working solution for detection of *Laf*, *Fla* genes in *Aeromonas*s pp.

Working solution	
Water	30 µl
Forward primer	5 µl
Reverse primer	5 µl
DNA	10 µl
Master mix	50 µl
Final volume	100 µl

Amplification was conducted using a master cycler eppendroff programmed with 35 cycler for Initial denaturation 95°C for 5 min. ,Denaturation for 94°C 25 sec., Anneling 55°C 25 sec.,Extention72°C 1min and final Extention 70 °C 5min. (Table 3).

Table (3): PCR program forfragment*Laf*, *Fla* amplification by the conventional methods.

Thermocycler conditions	Temperature (°C)	Time (min)
Initial denaturation	94°C	5min.
Denturation	94°C	25sec.
Primmer annealing	55°C	25sec.
Primmer extension	72°C	1 min.
Final extend	72°C	5 min.
Cycles number : 35 cycle		

Gel Electrophoresis**:** -PCR products and the ladder marker were resolved by electrophoresis on 2% w/v agarose gels. DNA samples were loaded in the tray of Gels and 100 bp marker was included in every gel and run in TBE(1X) buffer, Gels were stained with ethidium bromide (0.5 µgml-1) and analyzed using UV eliminator The molecular weight identification of resolved band was based on their correspondence to the ladder bands.

Results and Discussion

1- Analysis of Drinking Water Samples: - To estimate quality of drinking water, one thousand five hundred sixty seven samples were bacteriologically examined for fecal and totalcoliform, *E.coli* using MF technique, tube fermentation test and presence /absence method and PCR method were conducted. (853) water samples examined were all positive for the fecal and total coliform detect by conventional diagnosis methods, among them (519) samples were positive for fecal and total coliform and in the same time positive for *E.coli* which lied in the range of detection limit .This may be due to presence of fecal contamination from human origin from early period. Thirteen isolates of *Aeromonas* spp. Were isolated and identified from tap water from different parts of Baghdad area depend on morphological characters on the (ADA) agar which is selective media for isolation. Yellow bright colonies 1-1.5 mm in diameter, were tested for oxidase production and positive colonies were further characterized with conventional biochemical tests and Api 20E. Further tested for these isolates were for production of hemolysine , most isolates were hemolys in positive , our findings demonstrate that the clear zone or hallo zone appeared around the bacterial growth of environmental isolates of *Aermonas spp.* on sheep blood agar indicating the production of β-haemolysin (Figure 1) that came in accordance with the results obtained by [11] and [3] Fig (1).

Fig. (1): *Aeromonasi*so lates produce β hemolysine on blood agar.

2- Detection the pathogenesis ofthe *Aeromonas spp.*:- Which include two lines:-

Animal module and the effect of toxins on organs:-

After 5 h from inoculation, the group two of micewere killed for the detection of FAclinicalexaminations showed deterioration in health of the animals with presence of wetness around the anus corrodes, after post –mortemexamination the

intestines appeared red and inflated with the liquids this gave positive FA (≥100).After 24 hmice were sacrificed and the pathogenesis effects on different tissues were screened compared with control mice tissues.

Histopathological changes of gastric section presentedmild degeneration of gastric epithelial and slightly necrosis as shown in (Fig 2B). When compared with section of normal groups (Fig 2A).

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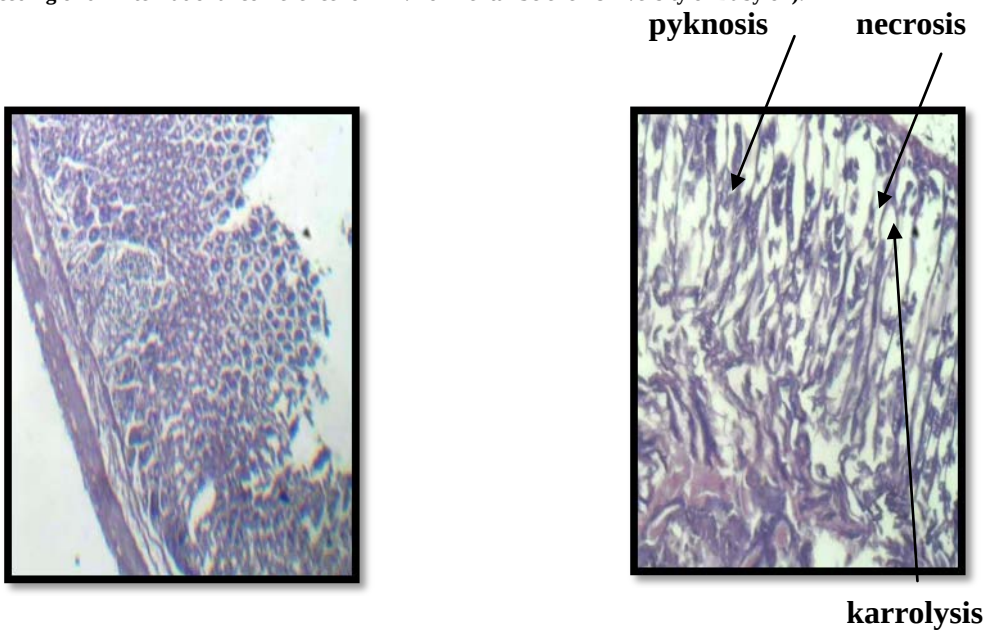


A:-Stomach(Control) B:-Stomach (infected)

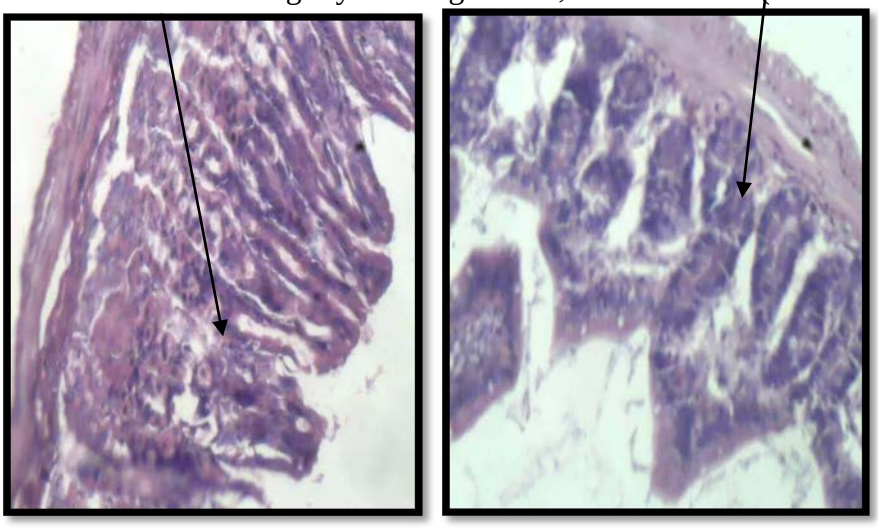
Fig. (2) Section of stomach for normal (control) group and infected mice with *Aeromonas spp.* (X200) (Hand E).

(A):-Section of gastric tissue of control group showing normal glandular group structure appearance.

(B):- Section of gastric tissue treated group showing mildto moderate degenerativechanges of gastric epithelial with necrosis of some cells (pyknosis, karryolsis).

While, small intestine suffered from damage with diffused sloughing in mucosa, some depress was located in the lumen of intestine, normal appearance of muscular mucosa with extensive damage in the villi (Fig. 3). This lead to propose the presence of virulence factor (s) one of them is toxin production with cytotoxic activity.

Normal structure villislightly shorting of villi, mild infiltrate (mononuclear cells)



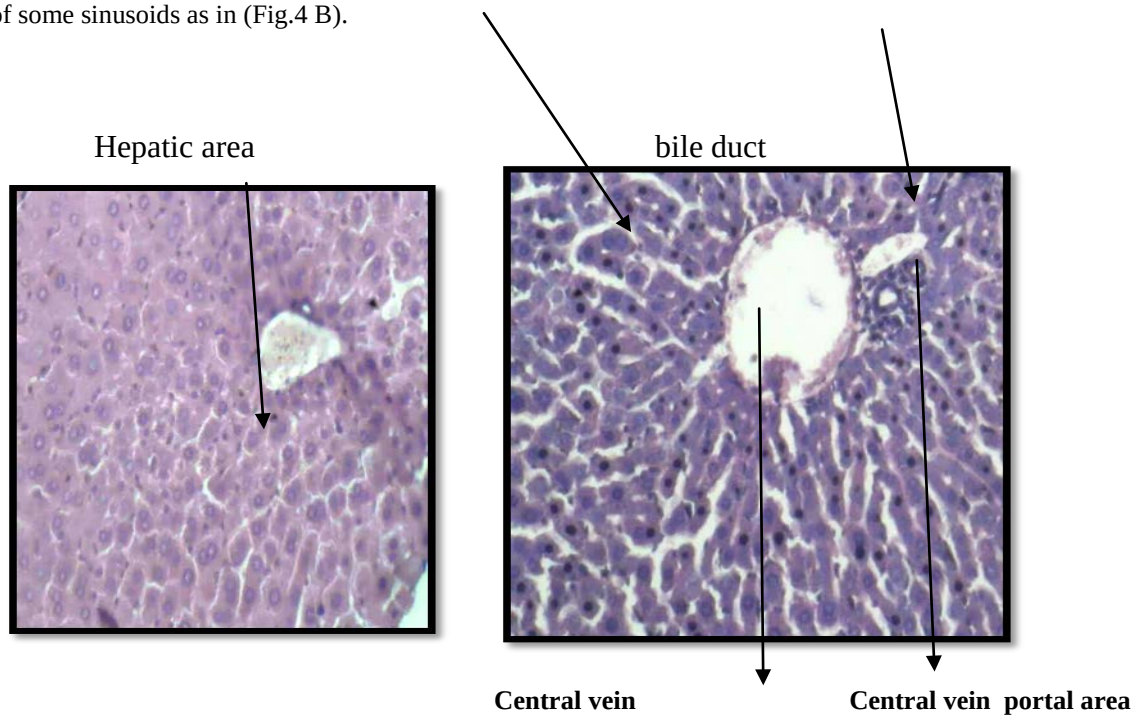
A: - Intestine (Control) B: -Intestine (Infected)

Fig (3): Section of intestine for normal (control) group and infected mice with *Aeromonas*(X200) (Hand E).

(A):- Section of normal intestine (control group) showed normal structure villi appearance.

(B):- Small intestine suffered from damage with diffused sloughing mucosa, and damage of villi appearance.

The liver tissue was examined and histopathological lesion of liver showed vacuolated of hepatocyte indicated to moderate degeneration , massive necrosis with infiltration of inflammatory cells and slightly loosed of the tissue architecture with dilated of some sinusoids as in (Fig.4 B).



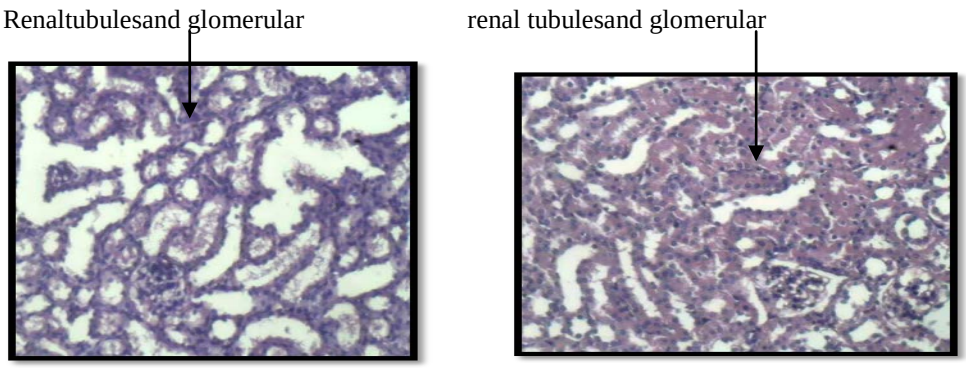
A:-Liver (Control) **B:-Liver (Infected)**

Fig (4):- Section of liver for normal (control) group and infected mice with *Aeromonas*(X200) (Hand E).

(A):-Section of liver (control group) showing normal structure appearance of hepatic cells and central vein.

(B):-Focal diseased area of hepatic cells necrosis withmononuclear inflammatorycells infiltrate near portal area.

The investigation results showed that kidney tissue apparently not changed, after post-inoculation after 24 h when compared with control section.



A:-Kidney (Control) **B:-Kidney (infected, looked like normal)**

Fig (5): Section of kidney for normal (control) group and infected mice with *Aeromonas*spp.(X200)(Hand E).

A-Normal structure appearance of renal tissue showing glomerular and renal tubules.

B-No effects appears on renal tissue.

Obviously, the results of this study came in agreement with other data such as [12] who examined the pathogenicity of *Aeromonas* spp. in the sealed – adult mouse model using live bacterial cells and toxigenic strain of *V.cholera* for comparison. Who demonstrated that of eighteen environmental isolates of*Aeromonas*spp.16 were positive for FA (≥ 100).

However , close to this results was reported in study carried out in Iraq [13]; [5] on non-O1 *V. cholera* isolated from surface water showed positive FA (≥ 100) for all isolates according to the histopathological lesions findings in current study, as a result of inoculate the *A. hydrophila* in laboratory animals .The present results confirmed the great microbial risks which associated with ingestion of water that contaminated with bacteria harboring virulence factors from human or

animal origin which is responsible for occurrence the pathogenicity by many mechanisms such as induce toxins ,adherences to the surface of smooth muscles , invasion and multiplication inside the tissues [14] .

Molecular level and PCR technique:-All samples were analyzed for conventional PCR for detection of virulence factors in isolated *A. hydrophila* using *Fla* and *laf*flagellin genes to diagnosis motility by polar and lateral flagellum which is responsible for *Aeromonas* swimming in liquid media and swarming in solid media respectively , most of isolates gave positive results for both these gene (fig. 6).

Fig (6): Conventional PCR for detection of *Laf* , *Fla* genes(504 pb) and(608 pb) , Lan:1,2,3, only positive for*Laf*primer and Lan: 6 was positive for *Fla* primer and Lan :- 4,5, 7,8,9,10,11,12 were negative for them, M:Marker DNA ladder.

Moreover, swimming motility mediated by the polar flagella is important in attachment to the surface and colonies the intestinal tract in case of clinical samples and constituent of bacterial biofilms in water distribution systems. Lateral flagella permit fast and local colonization, were bacteria multiply to form microcolonies Scoaris*etal*., [15] . Such conclusion was

supported by the work of Santons, *et al*[16] whom reported that isolates from environmental and clinical samples exhibit such genes, which have role in biofilm formation.

Several studies have shown that mutation in the genes involved in the synthesis of polar and lateral flagella lead to consistent reduction in both adherence and biofilm formation [17].

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