DOI : 10.35124/bca.2020.20.1.935

Biochem. Cell. Arch. Vol. 20, No. 1, pp. 935-942, 2020 | www.connectjournals.com/bca | *ISSN 0972-5075*

IN-VIVO PATHOGENICITY OF AEROLYSIN TOXIN OF *AEROMONAS HYDROPHILA* ISOLATED FROM DIARRHEA PATIENTS IN THI-QAR PROVINCE

Anwar Abid Al Kareem[1], Zainab Dakhil Degaim[2] and Abbas Dakheel Mutar[3]

[1]Department of Microbiology, College of Veterinary Medicine, University of Thi-Qar, Iraq.
[2]Department of Microbiology, College of Medicine, University of Thi-Qar, Iraq.
[3]Department of Basic Science, Collage of Dentistry, University of Thi-Qar, India.
e-mail : dr.abbas-mater@utq.edu.iq, zainab-d@utq.edu.iq

(Received 17 August 2019, Revised 23 December 2019, Accepted 29 December 2019)

ABSTRACT : ***Aeromonas*** **species are highly distributed in the world and causative agent for fish, animal and human diseases. The study concluded 255 fecal sampler (168 sample were collected from patient suffering from diarrhea and 82 sample from apparently healthy). The *Aeromonas hydrophila* isolates were identified depended on culture media and biochemical methods for I field *A.hydrophila*; the PCR technique used to detection thewhole forming Toxin (aerolysin) gene. The results revealed that 23 isolates of *A. hydrophila* were identified from diarrheal patients, while 18 isolates from apparently healthy. Aerolysin gene was detected in 9 isolates only. To determine the *in vivo* pathogenicity of *A. hydrophila*, forty two mice were used to detect LD_{50} value, then determined the histological changes of organs including intestine and liver were obtained for re-isolation and histopathological examination.**

Lethal dose of also demonstrated in mice was 0.9×1000000000. The microscopic examination of histopathological sections of intestine of infected mice after LD_{50} experiment showed that preserver of hyperplasia of lymphoid tissue with thickening in villi, congestion of central vein of liver, mild inflammation in periportal area, fatty changes and parenchymal cell of liver.

Key words : *Aeromonas hydrophila*, aerolysin toxin, lymphoid tissue, PCR technique.

INTRODUCTION

Aromonas species was an aquatic bacteria that founded in many natural environments and involved in a variety of human diseases (Janda and Abbott, 2010). Although, *Aeromonas* species were opportunistic pathogens for humans, some studies had shown that they may also act as primary pathogens for humans in a number of infections including; septicemia, wound infection, meningitis, pneumonia, hemolytic uremic syndrome, necrotizing fasciitis and gastro-enteritis (Cheng *et al*, 2004). The *Aeromonas* spp. recognized as human pathogens, include *Aeromonas hydrophila*, *A. caviae*, *A. veronii*biovar*sobria*, *A. veronii* biovar *veronii*, *A. jandaei*, *A. trota* and *A. schubertii* (Carnahan, 1993).

Aeromonas hydrophila was one of vital species among other species due to its recurrent relationship with human infection; also the virulent and non-virulent strains within this species had been described. Likewise *A. hydrophila* encompassing a numerous strains which differ in their pathogenic potential (Metz, 2015).

Bloom and Bottone (1990) stated that only subset of *A. hydrophila* strains can cause human diseases, necessitate the importance of scheme to differentiate the pathogenic from those non-pathogenic strains. A number of virulence factors derived from *A. hydrophila* had been proposed in an effort to explain the pathogenesis of infections. Also the Aerolysin was considered as an evident sign of the virulence of *Aeromonas* spp. (Heuzenroeder *et al*, 1999). Many studies on molecular biology of the virulence genes of diarrheagenic *Aeromonas* revealed that those strains harbored aerolysin toxin gene (*Aer*) are potential diarrheagenia in nature (Pollard *et al*, 1990). So the aim of this study were to determine the prevalence of Aerolysin positive *A. hydrophila* in diarrhea patients and study its pathogenicity in mice model.

MATERIALS AND METHODS

Sample collection

Two hundred and fifty five fecal samples, 168(67.2%) from patients with diarrhea and 82(32.8%) from apparently healthy were collected from different age

groups attending different hospitals in Thi-Qar province in period from August to November 2015. Fecal samples were collected by sterile swabs and transported to laboratory in Cary-Blaire transport medium.

Isolation and identification

A method described by Ghenghesh *et al* (2008) was adopted with some modifications briefly; Fecal swabs were inoculated in 9 ml of alkaline peptone water (APW) and incubated at 37°C for 24 h. A loopful of enrichment medium (APW) was streaked on MacConkey agar and blood agar and incubated at 37°C for 24 h. The colony morphology, microscopic and set of biochemical tests including; esculin test, indol test, oxidase test, catalase test, resistance to vibiostatic agent O/129, 6.5% NaCl tolerance and string test were used for identification and API20E was used for confirmation.

Detection of whole forming toxin (Aerolysin) gene by polymerase chain reaction

Genomic DNA was extracted using Gained Bacterial gDNA extraction kit (Thailand).

Oligonucleotide primer for detection the Aerolysin gene as following: forward: 5'-GCC TGA GCG AGA AGG T-3' and reverse 5'-CAG TCC CAC CCA CTT C-3') designated by Wong *et al* (1998) with product size 416bp was selected and its specificity was determined in previous studies (Oleiwi, 2013).

PCR was conducted using 50µl of PCR tube containing 5µl of template DNA, 1µl (15 picomol final concentration) of forward and reverse primer, 5µl premix and the remaining volume was completed by distal water. Amplification condition was: initial denaturation 95^0C for 5min, followed by 35 cycles of denaturation at 92^0C for 30sec, annealing at 53^0C for 30sec then extension at 72^0C for 2 min, final extension at 72^0C for 1 min. The products were visualized by adding 0.2µl of ethidium bromide staining using 1.5% agarose gel using 1x TBE buffer at 70V for 45-60 min.

***In-vivo* pathogenicity**

Median lethal dose determination

Fresh broth culture of *A. hydrophila* was centrifuged at 2000 rpm for 10 min. The pelted of bacterial cells were suspended in 1ml of normal saline, then bacterial suspension was centrifuged. The bacterial pellet at bottom of tube re-suspended with 1ml of normal saline and centrifuged again, finally 1 ml of normal saline was added to bacterial pellet and agitated gently. Ten fold dilutions was prepared from this suspension ranging from 10^{-1} to 10^{-6}. Viable count was conducted for each dilution.

Forty two white albino female mice were obtained from the animal house at the Science College in Thi-Qar University, Iraq. The mic were housed in standard metal cages and were divided into seven groups (6 rats/cage), each group was intragastrically administered with one of six dilution, while control group was administered with normal saline. The experiment was continuous for seven days and during this time was detected the dead and live mice were scored. LD_{50} value was calculated according to Reed and Muench (1938).

Experimental infection

Fifteen mice were divided into three groups (n=5), two groups were injected with *A. hydrophila* at infective dose (lower than LD_{50}) to study the pathogenicity of this bacteria without killing the mice and one group was injected with normal saline intragastrically as control; mice were sacrificed after six days post infection. Morbidity and mortality was observed during experiment period. Histological changes were studies of organs including intestine and liverwere obtained for re-isolation and histopathological examination.

RESULTS

Generally, the total isolation rate of *A. hydrophila* was 23 (9.2%), from which 18 isolates of *A. hydrophila* that isolated from diarrheal samples with percentage (10.71%) higher than the isolates from apparently healthy 5(6.1%). The statistical analysis showed a significant difference at (P = 0.05) as shown in Table 1.

All colonies showed β-hemolysis on blood agar and produced a large raised colorless colonies on MacConkey agar plates, which was indicative of *A. hydrophila* (Figs. 1 and 2). Microscopical examination revealed that Gve$^-$ bacilli. Biochemical identification showed all isolates were positive for indol, Voges-Proskauer, catalase and unable to grow at 6.5% NaCl broth, resistance to O/129 disc, negative for string test, the results of all tests were positive of *A. hydrophila*. The confirmed identification by API20E system showed the excellent identification of *A. hydrophila* (Fig. 3).

Detection of aerolysin gene

Aerolysin gene was detected only in 9(39.13%) of *A. hydrophila* isolates, the specific band of Aerolysin with expected product size (416 bp) presented in Fig. 4. Aerolysin gene was detected in *A. hydrophila* isolates recovered from diarrheal samples 7/23 (30.43%) higher than the isolates from apparently healthy persons 2/23(8.69%) with a significant difference at ($p<0.05$).

***In-vivo* pathogenicity**

Determination of LD_{50} and experimental infection

The results of this study revealed that LD_{50} of *A.*

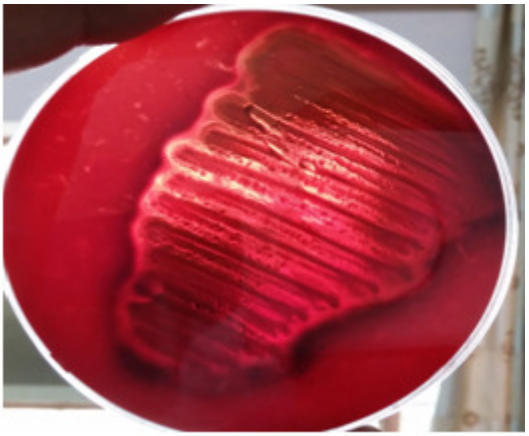

Fig. 1 : Beta hemolysis of *A.hydrophila* on blood agar

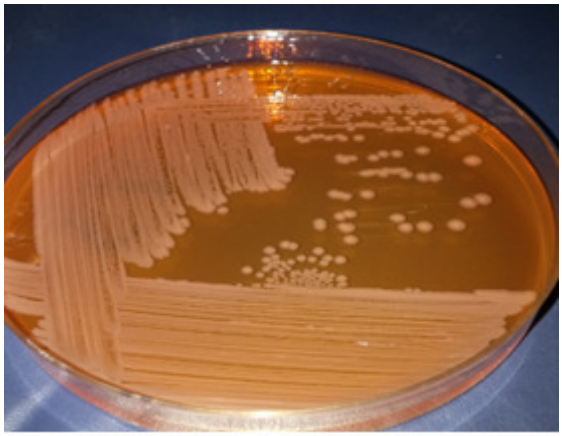

Fig. 2 : Growth of *A.hydrophila* on MacConkey agar medium.

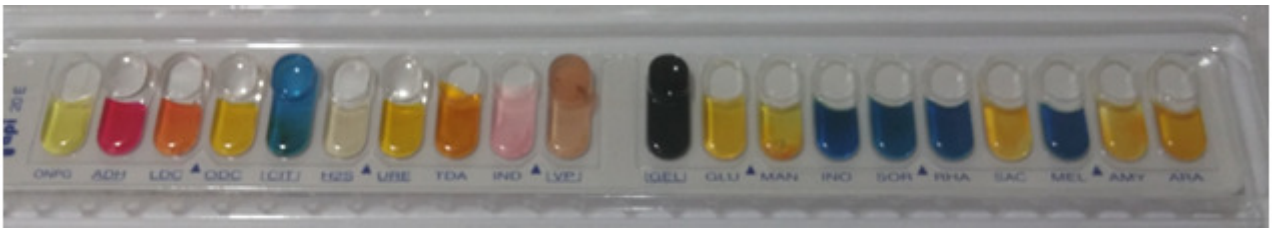

Fig. 3 : Results of API20E shows excellent identification of *A.hydrophila*.

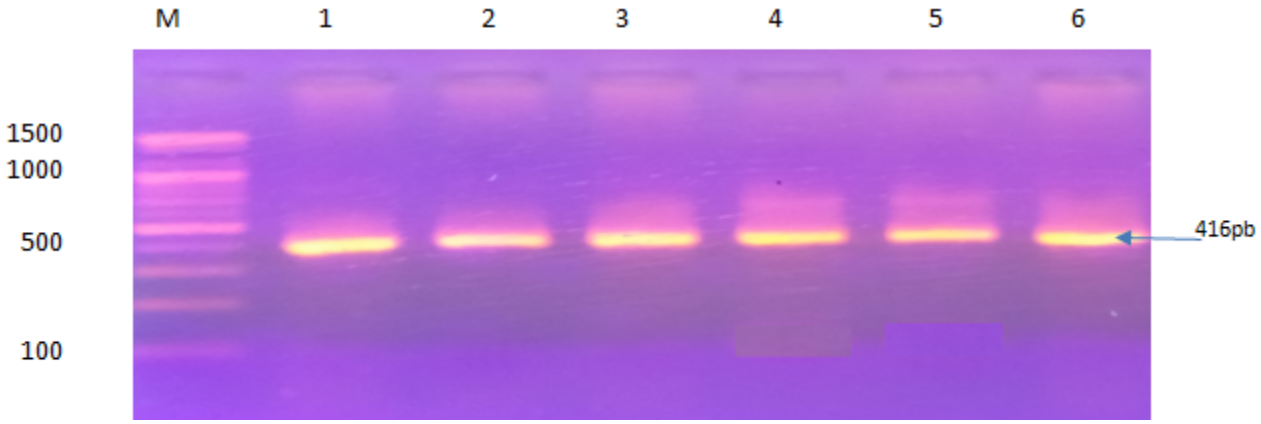

Fig. 4 : Agarose gel electrophoresis of Aerolysin gene amplificationin *A.hydrophila*, where M: ladder, 1-5: positive results.

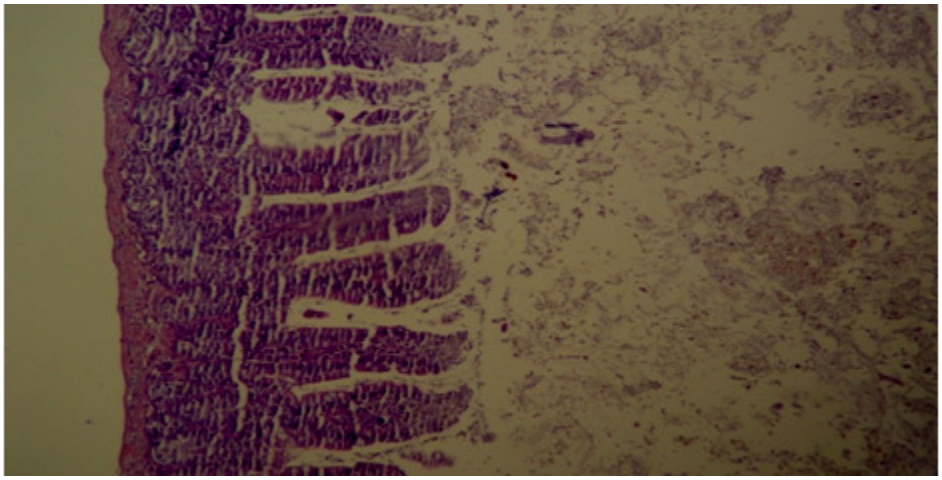

Fig. 5 : Histopathologcial section of intestine of mice in control group, showed no pathological changes (H&E X40).

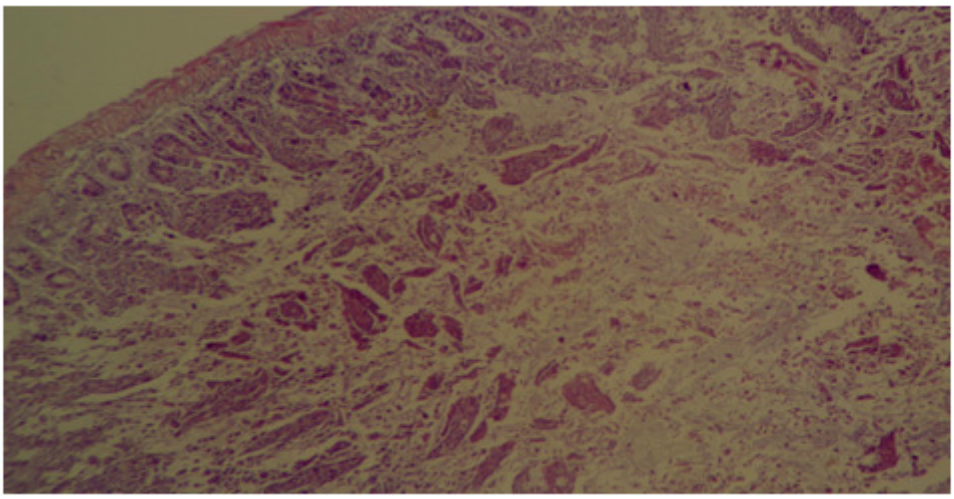

Fig. 6 : Intestine of infected mice shows epithelial sloughing of mucosal glands (H&E X40).

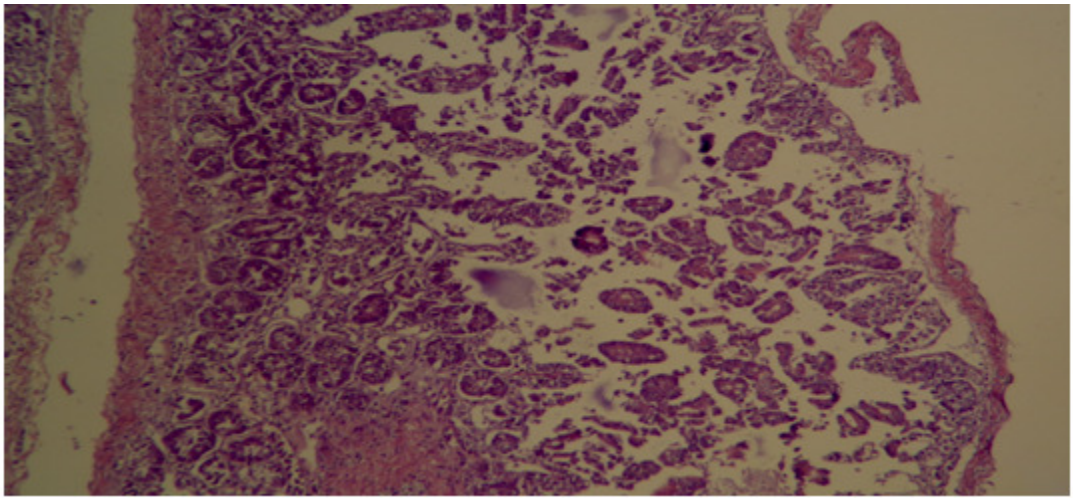

Fig. 7 : Intestine of infected mice shows epithelial sloughing of mucosal glands (H&E X40).

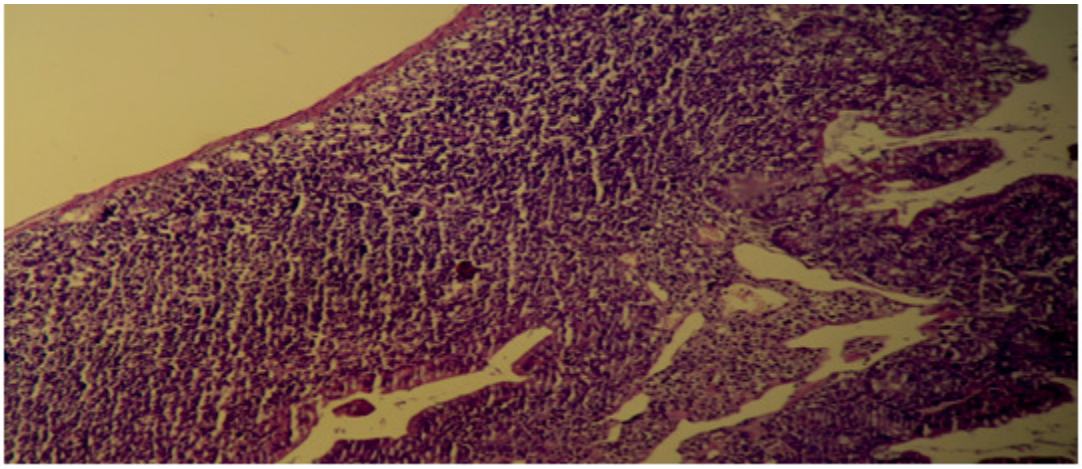

Fig. 8 : Intestinal wall of infected mice showed hyperplasia of the lymphoid tissue with thickening in the villi (H&E 5X).

hydrophila after seven days of injection was 6.9×10^9 as presented in Table 2. For studying the pathogenicity of *A. hydrophila*, the bacteria was administered intragastically at infective dose 6.9×10^6 at which no mortality was recorded. All infected mice were suffering lethality, loss of appetite, shaking and labored breath which were observed after 16 hours of administration, all these signs last for 3 days post infection. After 4 day post infection, all mice were apparently healthy except for one mouse, which dead at fourth day post infection. No diarrhea could be observed in all infected mice neither normal control. The bacteria had been re-isolated from

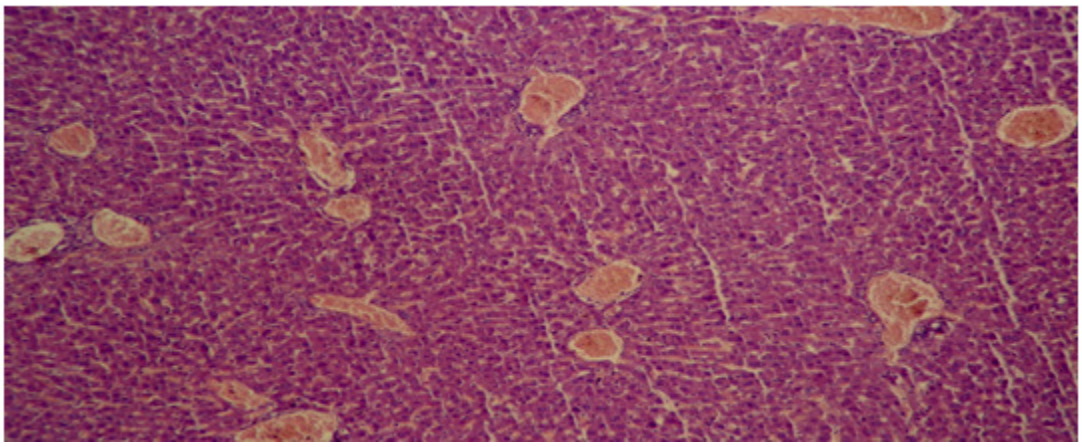

Fig. 9 : Liver of infected mice shows congestion of central vein (H&E X10).

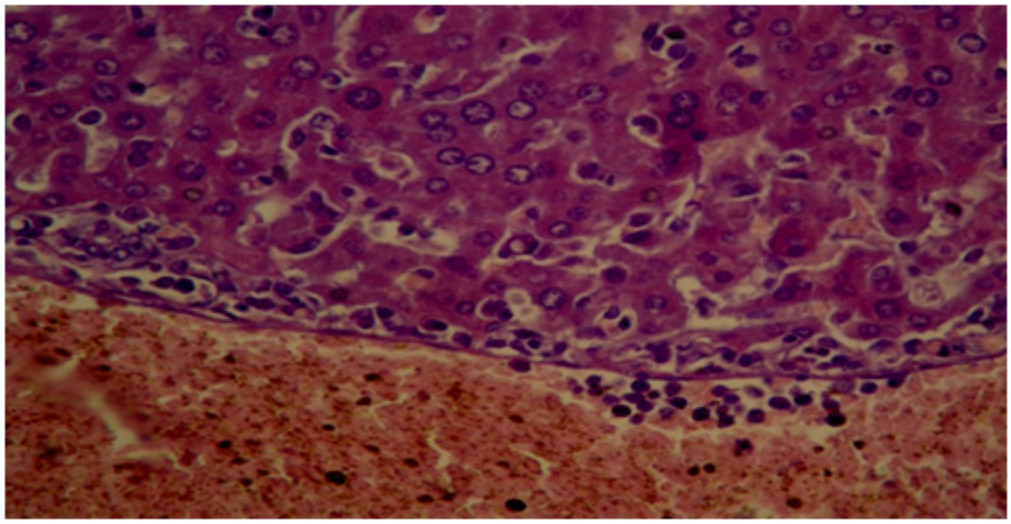

Fig. 10 : Liver of infected mice shows **A**) congestion of the portal vein **B**) mild inflammatory infiltrate in the periportal area.

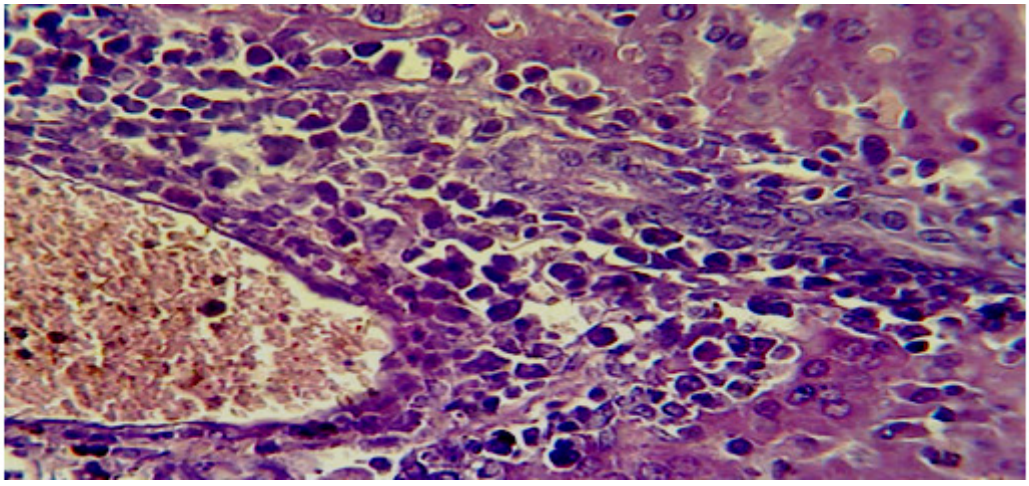

Fig. 11 : Showed infiltration of inflammatory cells neutrophils and mononuclear cells around congested blood vessels (H&E X10).

Table 1 : Recovery percentage of *A. hydrophila* from diarrhea and apparently healthy persons.

Nature of sample	No. of sample	*A. hydrophila*	P-Value
Diarrhea	168	18(10.71%)	P<0.05
Apparently healthy	82	5 (6.1%)	
Total	250	23(9.2%)	

internal organs (intestine and liver).

Histopathological examination

Light microscopic observation on section from control intestinal showed a normal structure (Fig. 5). Intestine of experimentally infected mice showed destruction of epithelial layer of mucosal gland (Figs. 6 and 7). Also, results of present study revealed that intestinal wall of

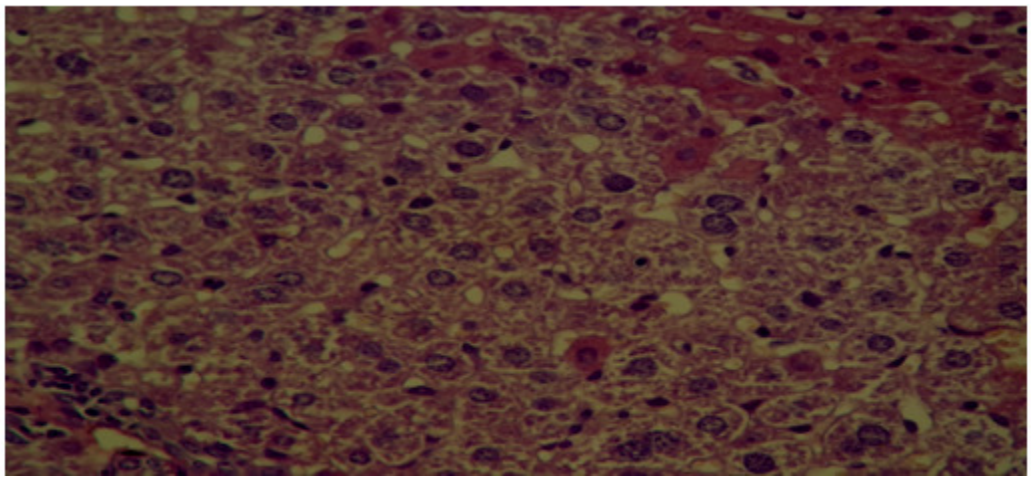

Fig. 12 : Liver section of infected mice showed fatty changes (H&E X10).

Table 2 : Results of median LD_{50} experiment of *A. hydrophila*.

Group(N=6)	Dilution	Dead	Survived	Accumulative value		
				Dead	Survived	Mortality%
1	10^{11}	6	0	20	0	100
2	10^{10}	6	0	14	0	100
3	10^{9}	4	2	8	2	80
4	10^{8}	3	3	4	5	44.5
5	10^{7}	1	5	1	10	9.09
6	10^{6}	0	6	0	16	0
Control						

$LD_{50} = 6.9 \times 10^{9}$

$$\text{Propotional distance} = \frac{\text{Mortality above 50\% – 50\%}}{\text{Mortality above 50\% – below 50\%}}$$

$$\text{Propotional distance} = \frac{80 - 50}{80 - 44.5} = 0.84 \text{Log } LD_{50} = 10^{9.84}$$

$LD_{50} = 6.9 \times 10^{9}$

infected mice showed hyperplasia of the lymphoid tissue with thickening in the villi (Fig. 8). Liver on the other hand showed congestion of central vein (Fig. 9), also the portal vein and the other changed recorded including mild inflammatory infiltrate in the periportal area, and fatty changes were observed in liver paranchymal cells (Figs. 10, 11 and 12). Also fatty changes were observed in liver paranchymal cells.

DISCUSSION

The role of *A. hydrophila* asenteric pathogen was not fully proven. Epidemiologically, this bacteria had been linked to acute diarrhea in some observations (Albert *et al*, 1999; Albert *et al*, 2000). The recent study revealed that a significant association between recovery of *A. hydrophila* as pure culture with diarrhea, this result was inconsistence with different works performed by Khardori and Fainstein (1988), Aslani and Hamzeh (2004). In general, results of current study concerning the overall recovery of *A. hydrophila* (9.2%) from human stool were in agreement with Kannan *et al* (2001), however the results of present study were higher than those reported by Taj Aldeen *et al* (2014).

Only 13% of *A. hydrophila* which recovery from diarrheal cases (Buchanan, 1984). Studies had revealed that the close association between the expression of a cell-free haemolysin by *Aeromonads* and Enterotoxigenic activity (Kudinha *et al*, 2000). The primary toxin haemolysins produced by some *Aeromonas* species was termed as "aerolysin" a heat-labile β-haemolysin, it possesses both hemolytic and enterotoxic activity expressed by many strains of *A. hydrophila* (Yu *et al*, 2005). Present study founded that Aerolysin positive *A. hydrophila* was detected in diarrheal patients at higher percentage than apparently healthy, in this respect results of this study were in agreement with Heuzenroeder *et al* (1999), Aslani and Hamzeh (2004).

However, phenotypically all strains were beta hemolytic, but only 9 strains showed positive for Aerolysin, this could be attributed to the fact that other toxins, which were not investigated in this study also possess hemolytic activity and confer hemolytic activity to those two isolates. Many studies supported that strains of *A. hydrophila* have more than one hemolytic toxins (Howard and Buckley, 1982). Also, it had been reported that *Aeromonas* could produce two types of lipase enzymes; lipase A1 and Phospholipase C and both have hemolytic activity (Merino *et al*, 1999).

Results of recent study showed that median lethal dose of *A. hydrophila* administered intragastrically was 6.9×10^{9}, which was in contrast (Daily *et al*, 1981; Janda and Kokka, 1991) whom recorded that *Aeromonas* LD_{50}

was 1.3×10^7 in mice infected intraperitoneally, this disagreement could be attributed to difference in rout of administration. Whoever, the results of this study was in line with Abood *et al* (2013).

Result of this study showed administration of *A. hydrophila* intragastrically did not cause diarrhea in mice, however the bacteria was re-isolated from the mice stool, indicative of intestinal colonization, thus results were in agreement with Sanderson *et al* (1996), who found that only streptomycin-treated adult mice could colonize *Aeromonas* in the intestine when the latter was administered intragastrically; however, no diarrheal symptoms were produced in this model.

In this study, intestinal sections showed destruction of epithelial layer of mucosal glands, neutrophils and mononuclear cells infiltration also sloughing of superficial epithelium and hyperplasia of lymphoid tissue. These finding were in agreement with Longa-Briceno (2008), Mansour *et al* (2014).

The changes observed in this study could in part be explained by the effect of endotoxin (lipopolysaccharides), the destruction of epithelial layer of mucosal glands and infiltration of inflammatory cells observed in this study were inagreement with Belal *et al* (2009), whoever aerolysin must also stimulate the infiltration of inflammatory cells as reported by Scheffer *et al* (1988), that the Aerolysin produced by *A. hydrophila* was the most potent stimulator of inflammatory mediator.

Histopathological changes of liver observed in this study were in agreement with Al-Saadi (2002), Abood *et al* (2013), whom found oral administration of *A. hydrophila* to mice at concentration (10^4) caused congestion of hepatic blood vessels and (10^6) caused congestion and hemorrhage in liver paranchyma.

CONCLUSION

We concluded, this study founded that *A. hydrophila* an important cause of diarrhea in Thi-Qar and that Aerolysin is an indicator of virulence that discriminate pathogenic strain, also the pathogenicity of these isolates confirmed by intragastrically of infected mice.

REFERENCES

Abood A S, Aali M A H and Jasim K A (2013) Histopathological effects of *A. hydrophila* on liver of white mice. *J. Bagh. Sci.* **2**(10), 260-268.

Albert M J, Faruque A S G, Faruque S M, Sack R B and Mahalanabis D (1999) Case-control study of enteropathogens associated with childhood diarrhea in Dhaka, Bangladesh. *J. Clin. Microbiol.* **37**, 3458–3464.

Albert M J, Ansaruzzaman M, Talukber K, Chopra A, Motiur R I, Faruque A S G and Mollby R (2000) Prevalence of enterotoxin genes in *Aeromonas* spp. isolated from children with diarrhea, healthy controls, and the environment. *J.Clin. Microbio*. **38**(10), 3785–3790.

Al-Saadi H Y F (2002) Pathological changes of polysaccharide extracted from *A.hydrophila*. *M.Sc thesis*, Science college, Baghdad University.

Aslani M and Hamzeh H S (2004) Characterization and distribution of virulence factors in *Aeromonas hydrophila* strains isolated from fecal samples of diarrheal and asymptomatic healthy persons, in Ilam, Iran. *J. Iran. Biomed.* **8**(4), 199 - 203.

Belal S K M, Abdel-Rahman A H, Mohamed D S, Osman H E H and Hassan N A (2009) Protective effect of pomegranate fruit juice against *Aeromonas hydrophila*-induced intestinal histopathological changes in mice. *J. Wor. Appli. Scien*. **7**(2), 245-254.

Bloom H G and Bottone E (1990) *Aeromonas hydrophila* Diarrhea in a Long-Term Care Setting. *J. Clini. Exper.* **38**, 804-806.

Buchanan R L (1984) The new pathogens. An update of selected examples. *J. Assoc. Food. Drug. Q. Bull*. **48**, 142-155.

Carnahan A M (1993) *Aeromonas* taxonomy: a sea of change. *J. Med. Microbiol. Lett*. **2**, 206-211.

Cheng N C, Horng S Y, Chang S C and Tang Y B (2004) Nosocomial infection of *Aeromonas hydrophila* presenting as necrotizing fasciitis. *J. Formo. Med. Associ*. **103**, 53–57.

Daily O P, Joseph S W, Coolbaugh J C, Walker R I, Merrell B R, Rollins D M and Lissner C R (1981) Association of *Aeromonas sobria* with human infection. *J. Clin. Microbiol.* **13**, 769-777.

Heuzenroeder M W, Wong C Y and Flower R L (1999) Distribution of two hemolytic toxin genes in clinical and environmental isolates of *Aeromonas* spp.: Correlation with virulence in a suckling mouse model. *J. FEMS. Microbiol. Lett*. **174**, 131-136.

Ghenghesh K S, Ahmed S F, Abdel El-Khalek R, Al-Gendy A and Klena J (2008) *Aeromonas*-associated infections in developing countries : A review. *J. Infec. Develop. Count*. **2**(2), 81-98.

Howard S P and Buckley J T (1982) Membrane glycoprotein receptor and hole-forming properties of a cytolytic protein toxin. *J. Biochem*. **21**, 1662-1667.

Janda J M and Kokka R P (1991) The pathogenicity of *Aeromonas* strains relative to genospecies and phenospecies identification. *J. FEMS Microbiol. Lett*. **90**, 29-34.

Janda J M and Abbott S L (2010) The genus *Aeromonas*: taxonomy, pathogenicity, and infection. *J. Clin. Microbiol. Rev*. **23**(1), 35-73.

Kannan S, Kanna P S, Karkuzhali K, Chattopadhyay U K and Pal D (2001) Direct detection of diarrheagenic *Aeromonas* from faeces by polymerase chain reaction (PCR) targeting aerolysin toxin gene. *J. Europ. Rev. for Med. and Pharma. Sci.* **5**, 91–94.

Khardori N and Fainstein V (1988) *Aeromonas* and *Plesiomonas* as etiological agents. *J. Annu. Rev. Microbiol*. **42**, 395-419.

Kudinha T, Tswana S A and Simango C (2000) Virulence properties of *Aeromonas* strains from humans, animals and water. *South. Afr. J Epidemiol. Infect*. **15**, 94-97.

Longa-Briceño A, Peña-Contreras Z, Dávila-Vera D, Rosa Virginia Mendoza-Briceño R and Palacios-Prü† E (2008) Experimental toxicity of *Aeromonas* spp. in mouse's small intestine: ultrastructural aspects. **33** (6), 457-460.

Mansour A M A, Zaki H M, Hassan N A and El-Nashar N A M (2014)

Phenotyping, virulence characteristics of *Aeromonas* species and the effects of essential plant oils as antimicrobial agents against pathogenic isolates from different sources. *Americ. J. Infect. Dis.* **10** (1), 21-35.

Merino S, Aguilar A, Nogueras M M, Regue M, Swift S and Tomas J M (1999) Cloning, sequencing, and role in virulence of two phospholipases (A1 and C) from mesophilic *Aeromonas* sp. serogroup O:34. *J. Infect. Imm*. **67**, 4008– 4013.

Metz J M (2015) Delineation of AeromonashydrophilaPathotypes by Detection of Putative Virulence Factors using Polymerase Chain Reaction and Nematode Challenge Assay. *M.Sc. Thesis*. Department of Molecular and Cellular Biology, Kennesaw State University.

Oleiwi S R (2013) Heavy Metal Resistance of *Aeromonas hydrophila* Isolated from Raw and Drinking Water in Baghdad City. *Iraq. J. Sci*. **57**, 1131-1135.

Pollard D R, Johnson W M, Lior H, Tyler S D and Rozee K R (1990) Detection of the aerolysin gene in *Aeromonas hydrophila* by the polymerase chain reaction. *J. Clin. Microbiol*. **28**, 2477–2481.

Reed L J and Muench H A (1938) Simple method of estimating fifty per cent endpoints. *Am. J. Hyg*. **27**, 493–497.

Sanderson K, Barnett T C and Kirov S M (1996) Type IV pilusadhesins and *Aeromonas* pathogenesis. *J. Microbiol. Aust*. **17**(4), A69.

Scheffer J, Konig W, Braun V and Goebel W (1988) Comparison of Four Hemolysin-Producing Organisms (*Escherichia coli*, *Serratia marcescens*, *Aeromonas hydrophila* and *Listeria monocytogenes*) for Release of Inflammatory Mediators from Various Cells. *J. Clin. Microbiol*. **26**(3), 544-551.

TajAldeen W R, Al-rubaiae A F, Al-bermani O K H and Naji N S (2014) PCR detection of putative hemolysin and aerolysin genes in *Aeromonas hydrophila* isolates from diarrhea in Babylon Province. *J. Nat. Sci. Res*. **4**, 41–47.

Yu H B, Zhang Y L, Lau Y L, Yao F, Vilches S, Merino S, Tomas J M, Howard S P and Leung K Y (2005) Identification and characterization of putative virulence genes and gene clusters in *Aeromonas hydrophila* PPD134/91. *J. Appl. Environ. Microbiol*. **71**(8), 4469-4477.

Wong C Y F, Heuzenroeder M W and Flower R L P (1998) Inactivation of two haemolytic toxin genes in *Aeromonas hydrophila* attenuates virulence in a suckling mouse model. *J. Microbio*. **144**, 291–298.