BEST: International Journal of Humanities, Arts, Medicine and Sciences (BEST: IJHAMS)
Vol. 1, Issue 2, Nov 2013, 15-22

ISOLATION OF OUTER MEMBRANE PROTEIN OF *AEROMONAS HYDROPHILA* RECOVERED FROM CHILDREN WITH DIARRHEA

NASEEM Q N. DUBAI[1] & AZHAR A. L AL-THAHAB[2]

[1]Department of Biology, College of Science, Al Muthanna University, Al Muthanna, Samawah, Iraq

[2]Department of Biology, College of Science, University of Babylon, Babylon, Iraq

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 2013from Al-Muthana public health laboratory in Muthana province - Iraq. All suspected isolates were screening by traditionally tests and then confirmed by Vitek 2 system and PCR technique(16S r RNA gene).The results showed that; there were 12 (4.08%) positive isolates of *Aeromonas hydrophila*. The SDS-PAGE method was used to analysis outer membrane proteins (OMPs) profile. The result revealed that, the OMPs molecular weight was around 44 KDa.

KEYWORDS: Identification *A. hydrophila*, Isolation of OMP, SDS-PAGE Analysis

INTRODUCTION

Diarrhea is a leading cause of childhood mortality and morbidity in developing countries and ranks among the most common causes of disease in children worldwide. Among bacterial etiologies of diarrhea, *A. hydrophila* is recognized increasingly as a clinically significant enteric pathogen. However, there are limited data on the prevalence and associated severity of diarrheal disease caused by *A. hydrophila* in many regions [1]Moreover a strong association between gastroenteritis and *Aeromonas* species has been shown in children, adults who are older than 60 years and in cases of 'traveler's diarrhea[2]

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[3]Virulence factors of *A. hydrophila*are present in two forms, cell-associated structures, and extracellular products. Among the cell-associated structures are pili, flagella, outer membrane proteins, lipopolysaccharide, and capsules. The major extracellular products include cytotoxic, cytolytic, hemolytic, and enterotoxic proteins [4]

The outer membrane of Gram-negative pathogenic bacteria has an important role in the interaction with hosts in the bacterial pathogenicity during adherence, uptake of nutrients from the host and eliminating host-defense mechanisms [5]

In previous study, methods based on restriction patterns of the polymerase chain reaction (PCR) amplified 16s rRNA genes were used for the identification of clinical strains of *Aeromonas spp*. Molecular techniques such as PCR and outer membrane based immunoassay have been used for detection and/or identification of *Aeromonas spp* from food or environmental or clinical samples [6].

Consequently, and from the above brief discussion *A. hydrophila* starts to have more space and attention from both Iraqi scientists and researchers alike. Therefore the current studyincluded isolation and identification of *A.hydrophila*from diarrhea collected samples, isolation and SDS-PAGE analysis of outer membrane protein (OMP).

MATERIAL AND METHOD

Samples Collection

A total of 294 stool samples were collected between December 2012 and February 2013 from patient children their ages were between (1month-6years) and suffering from diarrhea disease. These stool samples were obtained from Al-Muthana public health laboratory.

IDENTIFICATION OF *A. HYDROPHILA*

Cultural Methods

All samples were activated in APW media at 37 ^{0}C for 18-24h, and growing on culture media which are TCBS, MacCon key and Blood agar at 37 ^{0}C for 18-24hr [7].

Biochemical Identification

To confirm initial diagnosis of bacteria a manual biochemical tests were used such as catalase, oxidase, Indole, methyl red, simmone citrate, gelatin liquefaction and vogues-proskauer test[8].

Vitek System Identification

The Vitek 2 system assay has been used to confirm identification of *A.hydrophila*. This system performed according to the manufacturer's instructions (Biomerieux Company, France).

Molecular Identification

A polymerase chain reaction (PCR) technique was used to identify *A.hydrophila*by amplify genes of *16Sr RNA* gene from genomic DNA. DNA extraction from Gram negative bacteria was performed according to the genomic DNA purification kit supplemented by the manufacturing company (Geneaid/Taiwan). Gel electrophoresis has been used for detection of DNA by UV transilluminator[9]. The primers selection according to [8],[10] recommendations and used for diagnosis *A.hydrophila*. These primers synthesized by AccuOligo- Bioner Company, Korea, as shown in table (1).

Table 1: The Sequence of Forward and Reverse Primers

Primer Type	Primer Sequence	Product Size
Forward *16Sr RNA* - F	5-CCAGCAGCCGCGGTAATACG-3	300 bp
Reverse *16Sr RNA* - R	5-TACCAGGGTATCTAATCC-3	

PCR Mixture solution was according to information of manufacturing company(Master mix, Geneaid/Taiwan) and PCR Program conditions was listed in table (2)[8]. Ten µlstandard molecular weight of DNA ladder (marker) was loaded in first well on 1% agarose gel and each well has been loaded with 10µl of PCR product (DNA sample). Electrophoresis runs at 80 volt/cm for 1hr.

Table 2: Amplification Conditions

Steps	Temperature	Time	No. of Cycles
Initial denaturation	94 ^{o}C	3 min	30 cycle
Denaturation	94 ^{o}C	30 sec	
Annealing	52 ^{o}C	30 sec	
Elongation	72 ^{o}C	30 sec	
Final elongation	72 ^{o}C	10 min	

Isolation of Outer Membrane Protein (OMP)

The OMPs of *A. hydrophila* were prepared according to the method of [11] with few modifications.

From 2-3 litter of brain heart infusion broth was inoculated by the most virulence *A. hydrophila* isolate and incubated for 24- 48 h at 37 °C in a shaker incubator. After incubation period the bacteria was harvested by centrifugation at 6000 rpm for 15 min. The obtained pellet was washed twice by 40 ml of phosphate buffer saline (PBS, pH 7.2) and once in 40 ml of Hepes buffer (pH 7.4) then centrifuged at10000 rpm for 10 min. The cells were re suspended in 20 ml Hepes buffer and disrupted by sonication for 30 min at 10 watt an interval of 30 second with ice .Unbroken cells and cellular debris were removed by centrifugation at 4000rpm for 15 min at 4^{o}C.The resultant supernatant was then further centrifuged at 10000rpm for 1 hr at 4^{o}C.The supernatant was discarded and the pellet suspended in 20 ml of 2% triton X-100 and then incubated at room temperature for 30 min to solubilize the inner membrane .The suspension was then centrifuged at 10000 rpm for 1 hr at 4^{o}C. The supernatant was discarded and the pellet suspended in 1 ml of PBS (pH 7.2) and stored at -20^{o}C until use .Protein concentration of the OMP preparation was estimated by the Biuret method [12]

SDS-Page Analysis of Outer Membrane Proteins (OMPS)

The OMP analyzed by SDS-page was prepared according to the method of[13] using 12.5% (w/v) polyacrylamide in the resolving gel with few modifications. The OMP sample were diluted with sample buffer in ratio of 4:1 and heated at 95 ºC for 5 min.30 µl of the OMP sample containing 200 µg of protein was loaded in each lane of the gel. The gel was run at 150v for 6 hrs, and then stained with coomassie brilliant blue R-250 staining solution for overnight and then distained with distaining solution. The molecular weight (MW) of the protein was calculated by extrapolation of relative mobility of the unknown samples against that of standard molecular weight markers. SDS-PAGE analysis reagents are shown in table (3).

Table 3: SDS-Page Solution

Solution	Components of Solution	Amount
1. 1M TrisHCl (pH 8.8)	Tris.	12.1g
	Distilled water.Adjustment of pH 8.8 with concentrated HCl and 100 ml volume was made with distilled water.	50ml
2. 1M TrisHCl (pH 6.8)	Tris.	12.1g
	Distilled water.Adjustment of pH 6.8 with concentrated HCl and 100 ml volume was made with distilled water.	50ml
3. Electrophoresis buffer (pH 8.3)	Tris	3.025 g
	Glycine	14.413 g
	SDS	1.0 g
	Distilled water to make	1000 ml
4. Sample buffer (5X)	1 M TrisHCl (pH 6.8)	0.6 ml
	50% Glycerol	5 ml
	10% SDS (w/v)	2 ml
	1% Bromophenol blue	1 ml
	Distilled water	0.9 ml
5.polyacrylamide solution	Acrylamide	29.2 g
	Bisacrylamide	0.8 g
	Distilled water to make 100 ml	100 ml
6. Running Gel (12.5%)	Polyacrylamide solution	12.5 ml
	1M TrisHCl (pH 8.8)	11.2 ml
	Distilled water	6.2 ml
	10% SDS	0.3 ml
	TEMED	20 µl
	10% Ammonium per sulphate (w/v)	100 µl

Table 3: Contd.,

7.Staking Gel	Ployacrylamide solution	1.67 ml
	1M TrisHCl (pH 6.8)	1.25 ml
	Distilled water	7.03 ml
	10% SDS	0.4 ml
	TEMED	10 µl
	10% Ammonium per sulphate (w/v)	50 µl
8.Staining solution	Coomassie brilliant blue	0.15% in dark bottle
	Methanol	45%
	Acetic acid	10%
	Distilled water	45%
9.Distaining solution	Methanol	45%
	Acetic acid	10%
	Distilled water	45%

RESULTS AND DISCUSSIONS

Isolation of *A. hydrophila*

Two hundred ninety four of diarrheic stool samples were collected from Al-Muthana public health laboratory. The results showed that, there were 12 (4.08%) positive isolates of *A.hydrophila* (Figure 1). These results are almost agree with [14] were the highest prevalence of *Aeromonas* was observed in infants (< 1 years old) in Brazil. As well as, several authors reported that the high frequency of *Aeromonas* was found in infants and elder [15]. Moreover, [16] found out diarrhea disease that caused by *Aeromonas* was more frequently in children whom age between 1-3 years, in South India. Likewise, [17] reported that from one hundred twenty eight (128) diarrheic stool samples analyzed, 4 (3. 12%) were found to be positive for *Aeromonas hydrophila* in Nigeria.

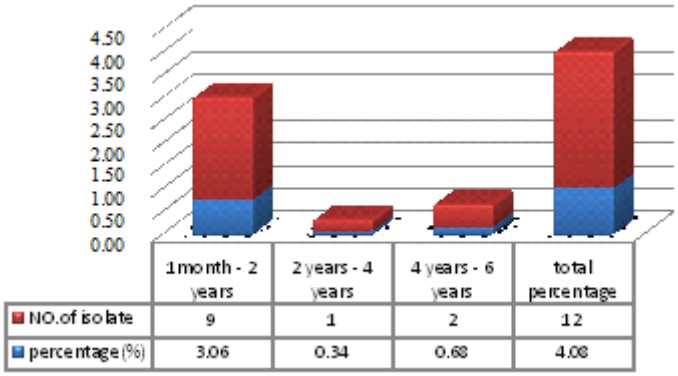

Figure 1: Frequency of *Aeromonas hydrophila* from Diarrheicstool Samples According to Children Patients Age

Identification of *A. Hydrophila*

*Aeromonas hydrophila*showed a yellow shine colour on TCBS agar, pale (non -lactose fermenters) on the MacConkey agar, smooth, convex, rounded, β-hemolytic colonies and pale white to grey colour on blood agar[8]. In terms of, initial biochemical tests *A. hydrophila* showed a positive result to each of catalase, oxidase, Indole, methyl red, simmone citrateand gelatin liquefaction. The current results of the biochemical tests in this study are almost finding in the other researchers reports [18],[19] While, *A. hydrophila* was gave variable results tovogues-proskauer. Vitek 2 system is an efficient biochemical testto confirm identification of *A. hydrophila* [19].The analytical profile index of this system has showed probability identification between (97%-99%) percentage.

In this study a polymerase chain reaction (PCR) technique was used toidentify *A.hydrophila*by amplify genes of *16Sr RNA* gene from genomic DNA of all *A.hydrophila* isolates. All isolates have given a positive results for *16Sr RNA*

(300)bp. The resultsillustrated in (Figure 2). In fact, [8] stated thatmost of *A. hydrophila* isolates shown a positive result to detected for (*16SrRNA*) gene. In addition to that, they reported the ribosomal mainly 16Sr RNA gene has confirmed to be a stable and specific molecular marker for the identification of *A. hydrophila* bacteria.

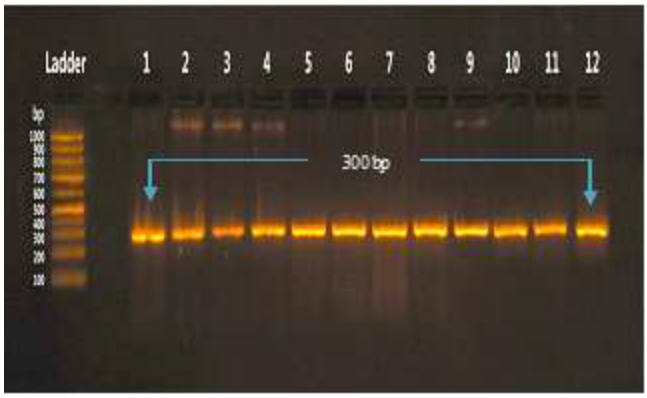

Figure 2: Agarose Gel Electrophoresis of PCR Amplified of *16S rRNA* Gene 300 bp of *Aeromonas hydrophila* Isolates for 1 hr at 80 Volt. Lane 1 DNA Marker (100bp Ladder). Lane 2,3,4,5,6,7,8,9,10,11,12,13 Amplify of 16Sr RNA Gene in *A. Hydrophila*

Separation and SDS-Page Analysis of OMP from *A. hydrophila*

The outer membrane (OMP) play an important role in reaction with hosts in the bacterial pathogenicity throughout adherence, parasite on the host and eliminating host-defense mechanisms[6]. Indeed, in this study OMP *A. hydrophila* was separated from the most virulence isolate. As well as, the protein concentration was estimated and gave 2.2 g\L. Moreover, SDS- page analysis of the OMP of *A. hydrophila* revealed that the molecular weight (MW) of the polypeptide band estimated by comparison with standard MW markers run parallel inrange 28-180 KDa, and the OMP was revealed 44KDa molecular weight (Figure 3).

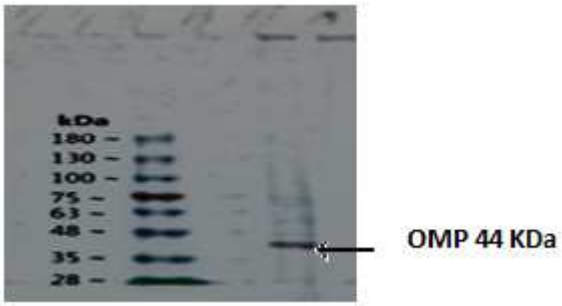

Figure 3: For 6 hr at 150 Volt. Lane 1: Protein Marker (180KDa Ladder). Lane 2: OMP 44KDa *A. hydrophila*

Different researchers noticed that major protein bands in the range of 30 and 45 kDa [20] and this finding is agree with the observe result of this study. As well as, likewise [21] reported that major proteins also ranged between 55 and 28 kDa. Moreover, in the study of OMP highly virulence group of strains, [22] indicated that major proteins of MW of 30 kDa and proteins are predominant. In fact, these variations are not unexpected due to the complex and diverse nature of *Aeromonas* spp**.** [20].

Therefore, further studies in Iraq need to be done on the area of investigate of OMP antigen ability to induce immunity response and make sure if it can be used as a potential vaccine to control *A. hydrophila.*

REFERENCES

1. Mansour, A. M., Elkhalek, R. A., ShaheenH. I., Mohammady, H.E., Refaey, S., Hassan,K., Riddle, M., Sanders, J. W., Sebeny,P. J., Young, S. Y. N. &Frenck, R. 2012. Burden of Aeromonas hydrophila –associated diarrhea among children younger than 2 years in rural Egyptian community. Journal Infection DevCtries 6(12): 842-846.

2. Sinha, S., Shimada, T., Ramamurthy, T., Bhattacharya, S.K., Yamasaki, S., Takeda, Y. & Balakrish, G.N. 2004. Prevalence, serotype distribution, antibiotic susceptibility and genetic profiles of mesophilic Aeromonas species isolated from hospitalized diarrhoeal cases in Kolkata, India. Journal of Medical Microbiology 53: 527-534.

3. Hochedez, P., Hope-Rapp, E., Olive, C., Nicolas, N., Beaucaire,G. &Cabié,C. 2010. Bacteremia Caused by Aeromonas hydrophila Complex in the Caribbean Islands of Martinique and Guadeloupe. The American Society of Tropical Medicine and Hygiene 83(5):1123–1127.

4. EPA, Environmental Protection Agency. 2006. Aeromonas: Human Health Criteria Document. EPA, Washington, DC.USA. Available at:-

   http://water.epa.gov/action/advisories/drinking/upload/2009_02_03_criteria_humanhealth_microbial_aeromonas-200603.pdf. [Accessed 2/8/2013].

5. Seltman, G. & Holst, O. 2000. The Bacterial Cell Wall. Berlin, Heidelberg and New York: Springer-Verlag.

6. Maiti, B., Raghunath, P., Karunasagar, I. & Karunasagar, I. 2009. Cloning and expression of an outer membrane protein OmpW of Aeromonas hydrophila and study of its distribution in Aeromonas spp. Journal of Applied Microbiology 107:1157–1167.

7. AL-Fatlawy, H. N. K. & AL-Ammar M. H. 2013. Molecular study of Aeromonas hydrophila isolated from stool samples in Najaf (Iraq). International Journal Microbiology Research Vol, 5, Issue 1, pp. 363-366.

8. MaccFadin, J. K. 2000. Biochemical test for identification of medical bacteria. (3rd ed .). Lippincott Williams and Winkins .AwolterKlumerCompany. Philadelphia Baltimor. New York.

9. Sambrook, J. &Rusell, D.W. 2001. Molecular cloning.A laboratory manual. 3rd ed. Cold Spring Harbor Laboratory Press, NewYork, USA.

10. Yogananth, N., Bhakyaraj, R., Chantchuru, A., Anbalagan,T. &MullaiNila, K. 2009. Detection of virulencegene in Aeromonas hydrophila isolated from fish samples using PCR technique. Global Journal Biotechnology Biochemical 4: 51–53.

11. Behera, T., Nanda, P. K., Mohanty, C., Mohapatra, D., Swaina,P., Das, P. K., Routray, P., Mishra, B. K. & Sahoo, S.K. 2010. Parenteral immunization of fish, Labeorohita with Poly D, L-lactide-co-glycolic acid (PLGA) encapsulated antigen microparticles promotes innate and adaptive immune responses. Fish & Shellfish Immunology 28: 320-325.

12. Tietz, N. W., Burtis, C. A., Ashwood, E.R. & Saunders, W.B. 1999. Text Book of Clinical Chemistry. 3rd Ed. 477-530.

13. Sachan, N., Agarwal, P.K. &Singh,V. P. 2012. Study on Outer Membrane Protein (OMP) Profile of Aeromonas Strains using SDS-PAGE. Research.Vet World Vol.5 (3): 173-177.

14. Guerra, I. M. F., Fadanelli, R., Figueiró, M, Schreiner, F., Delamare, A. P. L. Wollheim, C., Costa, S. P. O. & Echeverrigaray, S. 2007. Aeromonas associated diarrhoeal disease in south brazll: prevalence virulence factors and antimicrobial resistance. Brazilian Journal Microbiology 38: 638-643.

15. Janda, J.M. & Abbott, S.L. 2010. The genus Aeromonas, taxonomy, pathogenicity, and infection. Clinical Microbiology Reviews 23: pp. 35-73.

16. Meiyanti, S., Salim, O. C., Surjawidjaja, J. E. &Lesmana, M. 2010. Isolation and antibiotic sensitivity of Aeromonas from children with diarrhea. Universal Medicina Vol, 29. No.1.

17. Rogo, L.D., Attah, A., Bawa, E., Aliyu, A.M., Aliyu, M.S. &Gaiya, A.Z. 2009. Antimicrobial susceptibility pattern of Aeromoas Hydrophila among patients presented with diarrheal attending two teaching hospitals in northern Nigeria. Bayero Journal of Pure and Applied Sciences 2(1): 91 – 95.

18. Erdem, B., Kariptas, E., Cil, E., Isik, K. 2011. Biochemical identification and numerical taxonomy of Aeromonas spp. isolated from food samples in Turkey. Turkey Journal Biology 3 463-472.

19. Kivanc, M., Yilmaz, M., Demir, F. 2011. The occurrence of Aeromonas in drinking water and the Porsuk River. Brazilian Journal of Microbiology 42: 126-131.

20. Kuijper, E. J., Van-Alphen, L., Leenders E. &Zanen, H. C. 1989. Typing of Aeromonas strains by DNA restriction endonuclease analysis and polyacrylamide gel electrophoresis of cell envelops. Journal Clinical Microbiology 27: 1280-1285.

21. Santos, Y., Bandin I. &Toranzo, A. E. 1996. Immunological analysis of extracellular products and cell surface components of motile Aeromonas isolated from fish. Journal Applied Bacteriology 81: 585-593.

22. Dooley, J. G. S. & Trust, T. J. 1988. Surface protein composition of Aeromonas hydrophila strains virulent for fish: Identification of a surface array protein. Journal Bacterioogy 170: 499-506