Iraqi Journal of Veterinary Sciences

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Genetic diversity and clonal relatedness of Aeromonas hydrophila strains isolated from hemorrhagic septicemia's cases in common Carp (*Cyprinus carpio*) farms

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Article information

Article history:
Received July 8, 2020
Accepted August 27, 2020
Available online October 1, 2021

Keywords:
Aeromonas hydrophila
Fish farms
Genetic diversity
ERIC-PCR

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Abstract

The objective of this study was to determine molecular typing and comparison analysis of 24 *Aeromonas hydrophila* isolated from the diseased fish with hemorrhagic septicemia in freshwater ponds and cage in Mosul and Duhok cities, Iraq. A total of 24 *A. hydrophila* isolates that were collected from various fish ponds and cage, were used in this study. Identification of isolates was made by the standard microbiological and molecular methods. ERIC-PCR was done with different primers to establish the genetic relationship between strains. ERIC-PCR typing showed that 24 strains of *A. hydrophila* were classified into 11 ERIC types (genotypes). Genotypes 9 and 7 represented the most prevalent clone. All *A. hydrophila* strains that were isolated from the same fish were genetically diverse. There was minimal genetic similarity between some strains which were retrieved from the same geographical source area. Also, some isolates from different geographic source area were showed a 100% genetically similar. *Aeromonas hydrophila* was genotypically heterogeneous and clonally dispersed among different fish ponds and cage in Mosul and Duhok cities, Iraq. Besides, one fish can be infected with more than one strains of *A. hydrophila*.

DOI: 10.33899/ijvs.2020.127566.1511,

Introduction

Nearly every aquatic ecosystem has had *aeromonads*, including chlorinated drinking water, untreated sewage, groundwater and contaminated and unpolluted rivers (1,2). In addition, various domesticated animals may also have a role for the transmission of these bacteria through animal excretion (3). As a gastrointestinal and extra intestinal infection agent in humans, *Aeromonas hydrophila* was associated. Moreover, the primary cause of septicemia in many fish species is *Aeromonas hydrophila* such as common carp (*Cyprinus carpio*), tilapia (*Oreochromis niloticus*), hog (*Lachnolaimus maximus*), catfish (*Siluriformes*), salmon (*Salmo salar*) and many other freshwaters and marine species (4). One of the challenges in dealing with the risk of bacterial infection is to detect potential pathogens sources (5). The development of so-called bacterial source-tracking methods, tools to assign host source to environmental isolates of *A. hydophila* has been of significant interest (6). For these reasons, molecular epidemiology is necessary for controlling the spread of *A. hydrophila* between fish ponds (7). Recently, molecular typing methods are commonly used in microbial typing, leading to the rapid advancement of molecular biotechnology. Several studies have commonly used molecular typing methods for clinical and environmental *Aeromonas* strains as an epidemiological investigation (7-9). These include pulsed-field gel electrophoresis (PFGE), multi-locus enzyme electrophoresis (MLEE), and Enterbacterial repeated intergenic consensus-PCR (ERIC-PCR) (4,10-12). These techniques are precise, reproducible, and easy to analyze the genome of bacteria, and to research the classification and recognition of phylogenetics (4). In addition, there are many strategies for investigating bacterial molecular diversity. ERIC-PCR has

proven its predictive accuracy and is a fast and relatively simple technique which makes it useful for regular epidemiological studies (9,13,14). *Aeromonas hydrophila* typing using ERIC-PCR is of easy application, low cost, good sensitivity and repeatability, and is ideal for bacterial genotyping and molecular epidemiology surveillance (7,15). Unfortunately, there was only one study carried out in Iraq to determine the strain variation and investigate the clonal relatedness of *A. hydrophila* in clinical and environmental samples from diseased fish, which was used a phylogenetic tree analysis to compare between isolates of *A. hydrophila* (16). Therefore, this study was intended to investigate the genetic diversity and clonal relatedness of *A. hydrophila* strains isolated from diseased carps showing signs of hemorrhagic septicemia from different carp ponds and cage around Mosul and Duhok cities, Iraq.

Materials and methods

Bacterial strains and genomic DNA extraction

A total of 24 isolates of *A. hydrophila* were used in this study to investigate their clonal relatedness (cluster analysis) through ERIC-PCR fingerprinting. These isolates were previously recovered from lesions in different organs (liver, kidney, heart and lesions from the skin) of common carp showing signs of hemorrhagic septicemia farmed in different carp ponds and cage (including three ponds in Sumel district, one cage from Mosul dam and one pond in Khanke area) showed in (Table 1).

The isolated strains were identified by phenotypic methods (typical colonies on blood agar, pale colonies on MacConkey agar, Gram's stain, indole production test, oxidase test, catalase test and urease test) and also by molecular method (through PCR amplification of *gcat* gene) (17).

Some of those strains were obtained from the same fish but with different tissues. These isolates have been probably classified as genetically related in particular those isolated from the same fish and from the same geographical source area. All strains were grown in brain heart infusion broth (BHI) (Lab, M, UK) at 37ºC and the stock cultures were performed and stored at -20ºC in BHI supplemented with 25% (v/v) glycerol (18).

DNA samples were extracted by thermal extraction method according to Taha and Yassin (19).

Briefly, 100 µl of stock culture was inoculated onto MacConkey agar. Two to three 2-3 pure colonies were mixed with 200 µl of sterile double distilled water. For at least 15 s, the mixture was vortexed and directly heated at 95ºC for 10 min; the samples then cooled instantly by ice, the cooled suspension was centrifuged.

One hundred fifty µl supernatant was used as a template DNA for PCR. The purity and concentration of extracted DNA were examined using a nanodrop (Thermo Scientific, USA).

Table 1: The assigning of 24 A. hydrophila with their geographical source area and the fish organs

Strain No.	Strain ID	Geographical source area	Fish's organ
1	S1a	Sumel pond 1	Liver
2 [a]	S1b	Sumel pond 1	Liver
3 [a]	S1c	Sumel pond 1	Skin
4 [b]	S2a	Sumel pond 2	Skin
5 [b]	S2b	Sumel pond 2	Liver
6	S2c	Sumel pond 2	Skin
7 [c]	S2d	Sumel pond 2	Heart
8 [c]	S2e	Sumel pond 2	Liver
9 [c]	S2f	Sumel pond 2	Kidney
10	S3a	Sumel pond 3	Liver
11	S3b	Sumel pond 3	Liver
12	S3c	Sumel pond 3	Liver
13 [d]	S3d	Sumel pond 3	Kidney
14 [d]	S3e	Sumel pond 3	Heart
15	KHa	Khanke	Liver
16	KHb	Khanke	Liver
17	KHc	Khanke	Kidney
18	KHd	Khanke	Heart
19	KHe	Khanke	Heart
20	KHf	Khanke	Kidney
21	Ma	Mosul dam	Kidney
22	Mb	Mosul dam	Heart
23	Mc	Mosul dam	Kidney
24	Md	Mosul dam	Heart

The data of this table were derived from the previously published study (17). The same lowercase letter (a,b,c and d) parallel to strain numbers (in bold) indicates that these strains were isolated from the same fish. S: Sumel; KH: Khanke; M: Mosul dam.

ERIC-PCR fingerprinting

All *A. hydrophila* isolates were subjected to ERIC-PCR to identify similar strains and distinguish different strains using the primer sequences **(**ERIC1: 5'-ATGTAAGCTCCTGGGGATTCAC-3' and ERIC2: 5'-AAGTAAGTGACTGGGGTGAGCG-3') described by Versalovic *et al*. (20). The PCR assays were carried out in a total volume of 25 µl. Each reaction consisted of 2 µl primers for each 10 pmol, 12 µl of hot start premix (Genedirex, Taiwan), 2 µl of sample DNA 30-100 ng/µl and nuclease-free water 9 µl (Qiagen, Germany) up to 25 µl (21). The PCR reaction was carried out carried using PCR system 9700 GeneAmp (Applied Biosystem, USA) according to the PCR program used by Bakhshi *et al.* (14). The first denaturation was for 5 min at 94ºC, next with 35 cycles of repeated steps each of 94ºC for 1 min, 54ºC for 1 min, and 72ºC for 5 min. Finally, post PCR extension was done at 72ºC for 10 min. The Amplification of PCR products was loaded in 2% agarose gel prepared with 1× Tris-acetate-EDTA (TAE) buffer and stained by red safe DNA staining solution (GeNetBio, Korea). DNA ladder 100-bp (Genedirex,

Taiwan) was used as a molecular size standard. An image was captured for data analysis.

Data analysis

An image with 24 wells representing all isolates of *A. hydrophila*, was firstly recorded manually for the presence or absence of DNA bands in gel obtained from ERIC-PCR and then finally analyzed using the GelJ software version 2.0 (available at https://sourceforge.net/projects/gelj/) to generate dendrogram (22). The clustering of the isolates was performed based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analysis and Dice similarity coefficient. Isolates with a similarity coefficient equal to or above 90% (Similarity thresholds of ≥90%) were clustered as the same genotype (15). Strains were clustered according to their origin of isolation (the same or different geographical source area) and strains which were isolated from the same fish but with different organs.

Results

According to the ERIC-PCR fingerprinting analysis (Figures 1 and 2) and depending on the differences in the number and size of ERIC sequences found in each isolate, the results showed that the similarity among *A. hydrophila* isolates was between 57-100% and all isolates were grouped in to11 genotypes (1-11) according to 90% cut off similarity coefficient, in which genotypes 9 and 7 represented the most prevalent clone and its variants among the isolates comprising 11/24; 45.8% of total isolates. Six strains were clustered in genotype 9, followed by genotype 7 with 5 strains. On the other hand, each of genotype 1, 2, 5 and 6 have consisted of two strains. The remaining genotypes 3, 4, 8, 10 and 11 were included of single strain only (Table 2, Figure 2).

Interestingly, ERIC-PCR shows that all *A. hydrophila* strains that were isolated from the same fish were genetically diverse (all strains were showed a strong genotypic diversity) for examples, strain number 2 with 3 showed 57% genetic similarity, 87% similarity between strains 4 and 5, 77% similarity of strains 7, 8 and 9, 77% between strains 13 and 14, as indicated by the Dice coefficient and shown with lowercase letters (Figure 2).

Regarding the geographical source area, some strains that were isolated from the same geographical source area, were genetically diverse (there was a very little genetic similarity between some strains which were isolated from the same geographical source area), for example, strains Sumel pond 1 (S1a, S1b and S1c; 57% similarity), strains from Sumel pond 2 (S2a and S2d with S2b, S2c, S2f and S2e; 77% similarity), strains from Sumel pond 3 (S3b with S3a, S3c, S3d and S3e; 68% similarity), strains from Khanke area (KHa, KHb and KHc with KHd, KHe and KHf; 57% similarity) and finally strains from Mosul dam (Mc and Md with Ma and Mb; 64%). In addition, some isolates from different geographical source area were 100% genetically similar (S1b, S2a and S3a), (S2d and S2e with KHb), (S2c and S2e with KHa and KHc), (S1c with Md) and (KHe with Ma) (Figure 2, Table 3).

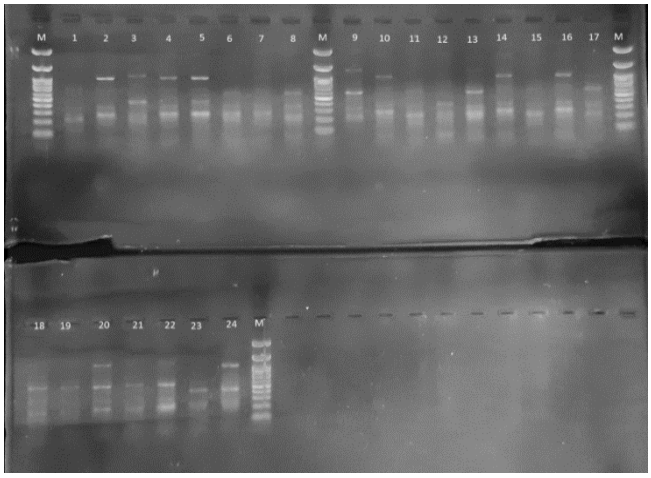

Figure 1: ERIC-PCR DNA fingerprint patterns of 24 *A. hydrophila* strains isolated from common carp with hemorrhagic septicemia. Lane 1-24 represent studied samples.

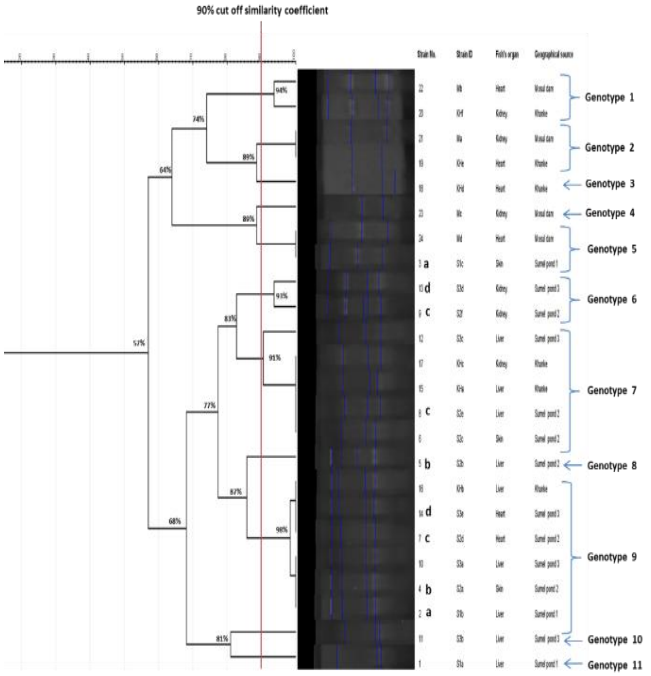

Figure 2: Dendrogram generated from ERIC-PCR showing banding pattern of 24 *A. hydrophila* strains isolated from common carp with hemorrhagic septicemia. The same lowercase letter (a,b,c and d) parallel to strain numbers indicates that these strains were isolated from the same fish.

Table 2: Genotypic pattern of 24 *A. hydrophila* strains isolated from diseased fish

Strain No.	Strain ID	Geographical area	Fish's organ	Genotypic pattern
20	KHf	Khanke	Kidney	Genotype 1
22	Mb	Mosul dam	Heart	
19	KHe	Khanke	Heart	Genotype 2
21	Ma	Mosul dam	Kidney	
18	KHd	Khanke	Heart	Genotype 3
23	Mc	Mosul dam	Kidney	Genotype 4
3	S1c	Sumel pond 1	Skin	Genotype 5
24	Md	Mosul dam	Heart	
9	S2f	Sumel pond 2	Kidney	Genotype 6
13	S3d	Sumel pond 3	Kidney	
6	S2c	Sumel pond 2	Skin	Genotype 7
8	S2e	Sumel pond 2	Liver	
12	S3c	Sumel pond 3	Liver	
15	KHa	Khanke	Liver	
17	KHc	Khanke	Kidney	
5	S2b	Sumel pond 2	Liver	Genotype 8
2	S1b	Sumel pond 1	Liver	Genotype 9
4	S2a	Sumel pond 2	Skin	
7	S2d	Sumel pond 2	Heart	
10	S3a	Sumel pond 3	Liver	
14	S3e	Sumel pond 3	Heart	
16	KHb	Khanke	Liver	
11	S3b	Sumel pond 3	Liver	Genotype 10
1	S1a	Sumel pond 1	Liver	Genotype 11

S: Sumel; KH: Khanke; M: Mosul dam

Table 3: Percentages of genetic similarity between some stains within the same and different geographic source area

Geographic source	Stains ID	Percentages of genetic similarity
Same geographic source	S1a, S1b and S1c	57%
	S2a and S2d with S2b, S2c, S2f and S2e	77%
	S3b with S3a, S3c, S3d and S3e	68%
	KHa, KHb and KHc with KHd, KHe and KHf	57%
	Mc and Md with Ma and Mb	64%
Different geographic source	S1b, S2a and S3a S2d and S2e with KHb S2c and S2e with KHa and KHc S1c with Md KHe with Ma	100%

S: Sumel; KH: Khanke; M: Mosul dam

Discussion

This study was done in order to find the answers about these two questions. The first one, is that at which extend there was a genetic similarity between *A. hydrophila* strains from fish farms in a same and different geographical source area and the second question is about; is it possible that the same fish can be infected with two genetically diverse strains of *A. hydrophila*. A high genotypic pattern (11 genotypes out 24 strains) found in this study. This is an indication that a high genetic diversity exists within this bacterium as confirmed by Aguilera-Arreola *et al.* (8). In addition, Shao-wu *et al.* (7), was also reported that there were a high genetic diversity of *A. hydrophila* strains from diseased carp, which they found three genotypic patterns among isolates of *A. hydrophila* in infected carp from different province in China.

This study found high genetic diversity among the strains isolated from the same geographical regions. This clonal variability of some isolates between the same fish pond and cage is an indication that there was a clonal expansion of certain strains of *A. hydrophila* has occurred in these fish farms (23).

Moyer *et al*. (24), reported that *A. hydrophila* strains collected within the same city exhibited genetic variability. Conversely, Algammal *et al*. (9), said that all retrieved *A. hydrophila* strains from the same geographic area have no genetic diversity and all of them have identical profiles.

In this study, all *A. hydrophila* strains that were isolated from the same fish were genetically diverse. Suggesting that the coexistence of many *A. hydrophila* clones in the water of these ponds and also this is an indication that the fish were not infected with clonally related strains (25). Unfortunately, there have been no studies conducted in fish that would prove this situation, except one study which was carried out in human patient and found that two *A. hydrophila* strains isolated from the same patient but from different tissues were genetically diverse (26).

Another point of interest in our study was the determination of 100% genetic similarity among some strains isolated from different geographical source area. Suggesting that there was a single source of infection may be existing for these fish ponds and cage. Genetic stability between different strains can be related to physiological adaptation to the environments that they exist (27). Aguilera-Arreola *et al*. (8), also found some strains of *A. hydrophila* isolated in different geographical locations, were clonally related since they displayed identical profiles 100% similarity. In this study, there was a high genetic diversity between stains which were isolated from fish cage in Mosul dam, when compared to the similarity of strains within fish ponds, this could be due to the differences in microbial contents of these two environments which in turn may be due to the presence of open system in fish cages that can be freely accessed by genetically diverse bacteria through continuous water re-circulation (28).

Conclusion

The data from this study found that *A. hydrophila* was genotypically heterogeneous and clonally dispersed among different fish farms in Mosul and Duhok cities, Iraq. Also, this study shows that one fish can be infected with more than one strains of *A. hydrophila.*

Acknowledgements

This study was supported by Duhok Research Centre (DRC), College of Veterinary Medicine, University of Duhok, Iraq.

Conflict of interest

No conflicts regarding publication of this manuscript.

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التنوع الجيني وعلاقة مجاميع لعزلات الايروموناس هايدروفيليا المعزولة من حالات الحمى النزفية البكتيرية في مزارع الأسماك الكارب الشائع

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الخلاصة

الهدف من هذه الدراسة هو تحديد التمييز الجيني والمقارنة بين أربعة وعشرين عزلة من جراثيم الايروموناس هايدروفيليا والمعزولة من الأسماك المصابة بالحمى النزفية البكتيرية في أحواض وأقفاص المياه العذبة في محافظتي دهوك والموصل، العراق. تم استخدام أربعة وعشرين عزلة من جراثيم الايروموناس هايدروفيليا التي تم جمعها من أحواض وأقفاص مختلفة في هذه الدراسة. تم تصنيف العزلات من خلال الطرق البكتيرية والجزئية القياسية. تم استخدام تفاعل السلسلة المتبلمرة للتسلسل التكراري بين الجينات للجراثيم المعوية مع مختلف البادئات لتحديد العلاقة الجينية بين العترات. أظهرت نتائج تفاعل السلسلة المتبلمرة للتسلسل التكراري بين الجينات للجراثيم المعوية أن الأربعة وعشرين عترة من جراثيم الايروموناس هايدروفيليا صُنفت إلى أحد عشر تسلسل تكراري بين الجينات للجراثيم المعوية اعتماد على نتائج تفاعل السلسلة المتبلمرة للتسلسل التكراري بين الجينات للجراثيم المعوية. الأنماط الجينية ٧ و٩ كانت الأكثر انتشاراً. كان التنوع الجيني موجوداً في كل أجناس جراثيم الايروموناس هايدروفيليا التي عُزلت من نفس الأسماك. بينما كان هناك القليل من التشابه الجيني بين بعض العترات التي عزلت من نفس المنطقة الجغرافية. فيما لوحظ ان بعض العزلات من مختلف المناطق الجغرافية أظهرت تشابه جيني وبنسبة ١٠٠%. بينت الدراسة أن جراثيم الايروموناس هايدروفيليا غير متجانسة جينياً بين أحواض وأقفاص الأسماك في محافظتي دهوك والموصل ويمكن أن تصاب السمكة الواحدة بأكثر من عزلة من جراثيم الايروموناس هايدروفيليا.

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