DETECTION OF THE MOST IMPORTANT PATHOGENIC BACTERIA AFFECT EXTERNAL ORGANS OF CYPRINUS CARPIO IN WASIT PROVINCE

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ABSTRACT

The present study was aimed to find the most common causative agents relates to skin ulceration in most common fish farming (ponds and cages) affects *Cyprinus carpio* in Wasit province harvested from three different regions. The laboratory isolation and detection of bacteria in this study was carried out in medical laboratory techniques in Middle Technical University and Central Public Health Laboratory in Baghdad from September to December 2020. For this study 240 infected common carp *Cyprinus carpio L.* were used, 120 samples from cages and 120 samples from ponds. Samples were taken from three regions, which represented the north, central, and south of the governorate. Samples from skin ulcers were taken from affected areas in the body of the fish, skin, gills and fins, then transporting to the laboratory to perform bacterial culture. The present study illustrates that the common causative agent of ulceration in fish skin, gills and fins was bacteria with most detected bacteria were *Aeromonas hydrophila* (56.7%) in cages and (37.5%) in ponds, followed by *Pseudomonas aeruginosa* (40.83%) in cages and (35.08%) in ponds, and *Citrobacter freundii* (41.66%) in cages and (25.8%) in ponds through the months including in this study. In conclusion, the most important pathogenic bacteria related to skin ulceration in both cages and ponds in all three locations in Wasit province were *Aeromonas hydrophila, Pseudomonas aeruginosa* and *Citrobacter freundii.* The infection rate was higher in cages compared to ponds because of increase pollution by sewage and organic compound and due to stress condition resulting from higher stoking density.

Key words: fish- carp- skin lesion- *Aeromonas hydrophila - Pseudomonas aeruginosa - Citrobacter freundii*

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الكشف عن اهم البكتيريا المسببة للأمراض والتي تصيب الأعضاء الخارجية لاسماك الكارب في محافظة واسط

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المستخلص

أجريت هذه الدراسة للكشف عن العوامل المسببة لتقرح الجلد في الأحواض والأقفاص لأسماك الكارب الشائع *Cyprinus carpio* في محافظة واسط والتي تم اخذها من ثلاث مناطق مختلفة. تم إجراء العزل والكشف المختبري عن البكتيريا في هذه الدراسة بتقنيات مختبرية طبية في الجامعة التقنية الوسطى ومختبر الصحة العامة المركزي في بغداد من أيلول إلى كانون الاول 2020. في الدراسة الحالية تم استخدام 240 من أسماك الكارب *Cyprinus carpio* L.و المصابة بالافات الجلدية بواقع 120 عينة أقفاص و 120 عينة من الأحواض. وأخذت عينات من ثلاث مناطق تمثل شمال ووسط وجنوب المحافظة. تم أخذ عينات من قرح الجلد من المناطق المصابة في جسم السمكة (الجلد والخياشيم والزعانف) ونقلها إلى المختبر لإجراء الزراعة البكتيرية. أظهرت نتائج الدراسة أن العامل المسبب الرئيسي للتقرح الخارجي للجلد والخياشيم والزعانف هو عدوى بكتيرية، وأن الأنواع البكتيرية الرئيسية كانت *Aeromonas hydrophila* بنسبة 56.7% في الاقفاص و 37.5% في الاحواض, و*Pseudomonas aeruginosa* بنسبة 40.83% في الاقفاص و 35.08% في الاحواض, و أخيرا *Citrobacter freundii* 41.66% في الاقفاص و 25.8% في الاحواض خلال الاشهر المتضمنة في الدراسة الحالية (من شهر ايلول الى كانون الاول). في الختام ، فإن أهم أنواع البكتيريا المسببة للأمراض المرتبطة بتقرح الجلد في كل من الأقفاص والاحواض في جميع المواقع الثلاثة في محافظة واسط هي *Aeromonas hydrophila* و *Pseudomonas aeruginosa* و *Citrobacter freundii* . كان معدل الإصابة أعلى في الأقفاص مقارنة بالاحواض بسبب زيادة التلوث بمياه الصرف الصحي والمركبات العضوية وبسبب ظروف الإجهاد الناتج عن ارتفاع الكثافة العددية.

الكلمات المفتاحية: أسماك، كارب، التقرحات الجلدية، ايرومونس هايدروفيلا، سيدومونس ارجينوزا، ستروبكتر فرنداي
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INTRODUCTION

Production of fish, gradually developed within the world also as in Iraq, the history of fish farming in Iraq are often traced back to the mid of last century (2). The most sorts of fish available within the fish farms of Iraq are common carp *Cyprinus carpio* L., silver carp Hypophthalmichthys molitrix and grass carp Ctenopharyngodon idella (19). Fish diseases are one of the most important problems in fish farm (8), fish farms are suffering from many pathogenic microorganisms including bacteria, virus, parasite and protozoa (24, 9) and these farms could even be suffering from non-infectious diseases (19). Skin disorders in fish are especially harmful and any surface injury to the skin makes fluid balance harder and should cause circulatory malfunction, therefore the skin layers are extremely important protective barriers for fish, and therefore the mucus allows fish to slide through the water more easily, so less energy is employed while swimming, also there are several protective compounds within the mucus that protect the fish from bacteria and other organisms within the water (21). Skin ulceration in fish can has many various aetiologies, including infectious agents, toxins, physical causes, immunologic causes, nutritional and metabolic disturbances (13). Fish exactly swim during a sea of pathogens. Thus, any gap within the normal barrier function of the skin can allow colonization of the skin by infectious organisms, or invasion by microorganisms that normally colonize the skin, differential diagnoses for skin lesions in fish should include fungi, virus, bacteria and parasitic organism (13). The aim of this study was isolation and diagnosis of the infectious agents of disease of the skin of the common carp fish at Wasit province.

MATERIALS AND METHODS

Sampling Area

The sampling area (Figure 1) included mud ponds and floating cages for fish farming from three main regions in Wasit provence. North of Wasit, Al-Suwaira district, Al-Shajiriya village, central area, Kut district, Al-Alkaya village, south of Wasit province, Alhay district, Alzarkan village. The infected fish, which had external ulcers on the skin, gills and fins, were placed in a container provided with air flow source and delivered to the laboratory. Swabs were taken by loop full and implanted on the culture media. The samples were taken from external surface of infected fish including skin, gills, and fins.

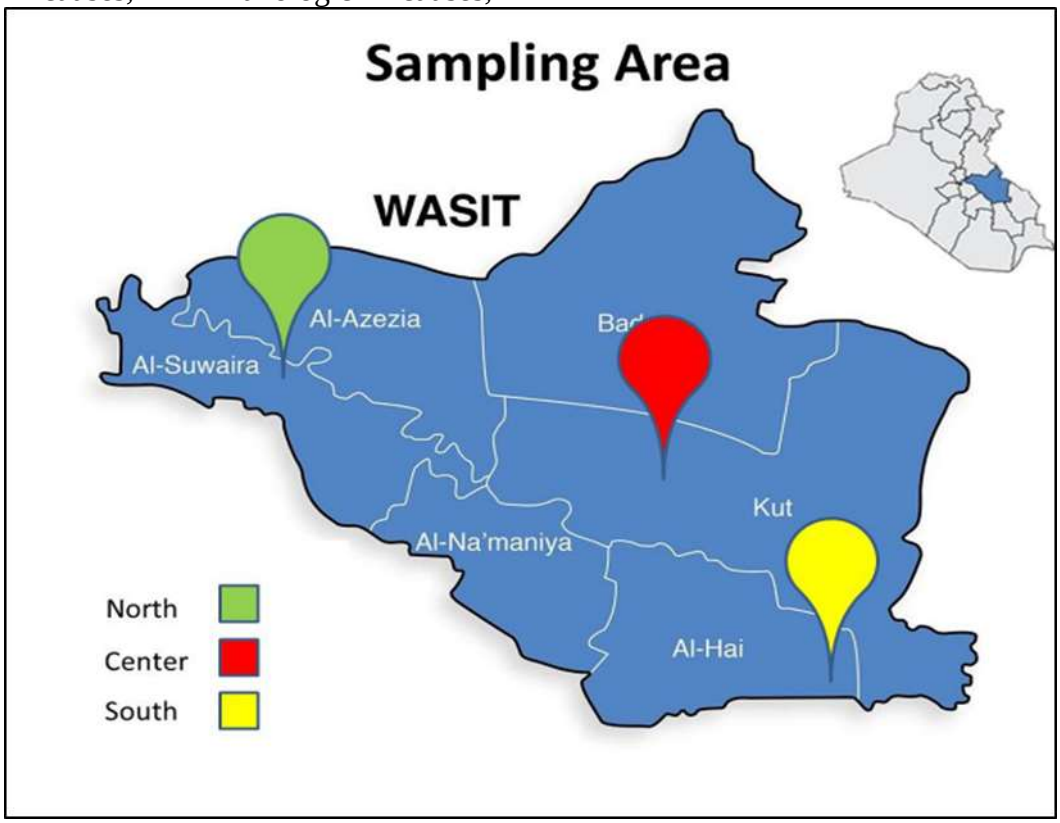

Figure 1. Wasit province map showing the different sampling area were the sample collected

Cultures Medias

The cultures medias were used in present study are Brain Heart Infusion Agar (BHIA), Brain heart infusion broth (BHIB). Nutrient broth, Blood agar, MacConkey Agar, Muller – Hinton agar, Thiosulfate-citrate-bile salts-sucrose agar, Triple Sugar-Iron Agar, Motility medium all prepared according to the company's instructions (23). Preparation of reagents and solutions including, Hydrogen peroxide (H_2O_2), N,N,N,N-tetramethyl-P-phenylenediamine Dihydrochloride (oxidase reagent). Formalin solution (10 %), Normal saline, Turbidity standard (McFarland) according to protocol described by Procop et al. (23). This study was conducted in Wasit governorate, fish infected external lesion (skin, gill and fins) samples were collected during the period from September to December 2020. The study was current in medical laboratory techniques in Middle Technical University and Central Public Health Laboratory in Baghdad. A total of 240 samples (80 sample from central Wasit province and the same number from north and south through four month) during a period from sptember, October, November and December 2020, samples were obtained, from Al- Sawira (Alshajiria), Al- Kut (Aleilkaya) and South Alhii (Alzarkan).

Isolation and identification isolated bacteria

Culturing the samples: Swap sterile sample placed in a test tube containing nine ml of BHIB, mixed leave it for 24 hr in incubator at 37ºC. Then MaCconkey agar, SS agar, mantoil salt agar were streaked by loopful from the broth and incubated at 37ºC for 18-24 hours. pink colonies, lactose ferment were transfer to TCBS agar and Blood medium and incubated at 37ºC for 18-24 hours initially, suspected colonies were sub culturing into MaCconkey agar for more identification.

Gram's stain

Bacterial smear was prepared by placed a loopful of distal water on a clean glass slide, then sterile the loop on flame and after cooling a loopful of colony transfer to the drop of water, mix well and dry by air. Fixation by passing the slide through Bunsen burner flame two to three times, stained with Gram's stain and examined under oil immersion lens of light microscope (10).

Biochemical tests: Biochemical tests were done according to Procop et al. (23).

Catalase test

Nutrient agar was used to detect bacteria by apply a single colony on a clean slide, then were added 1-2 drops of 3% H_2O_2 reagent and the development of O_2 bubbles indicated a positive result.

Oxidase test

It was made by place three drops of freshly prepared oxidase reagent into filter paper. Using a sterile wood rod to pick up a single colony of test organism and smear it on the filter paper. Positive result is consider when color of colony converted to dark purple within 2-10 seconds indicates a positive result.

Triple Sugar Iron test

Suspected colonies were stabbed into the tube bottom and streaked across the slant surface. The tube was incubated at 37ºC for 24 hours. A positive result that gives alkaline/acid reaction, without black precipitation and the formation of gas bubbles.

Motility test

Semi-solid medium was inoculated with tested bacterial culture by stabbing, incubated at 37ºC for 24-48 hours, motile organism recognized by movement faraway from the stab line or a hazy appearance through the medium. Identification of isolate by Api 20 E system. The bacterial suspension was prepared from purified isolated colonies by utilizing API suspension medium and therefore the turbidity was adjusted to 0. 5 McFarland tube (1-1.5x108 CFU/ml). By employing a sterile Pasteur pipette, the bacterial suspension was transferred to the 20 microtubes and inoculated consistent with the manufactures' instructions, and incubation for 24 hours at 37ºC, the isolates were identified by utilizing the numerical coding of the API system for confirmatory identification at species levels.

Maintenance of bacterial isolates

For short storage, the pure isolated bacterium was kept after the purification by streaking slants of BHIA and incubated for 24 hours at 37ºC, then slants were kept at 4ºC for a few weeks. For long storage, the pure isolated bacterium was inoculated in BHIB containing 15% glycerol and incubated for 24 hours at 37ºC, then maintained frozen for several months (13). Statistical analysis was

performed using SPSS (Statistical Package for the Social Science version 21). Chi2 test were performed to assess significant difference among means. $P \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The external lesion of the infected fish has huge number of hemorrhagic spots on the fish's body, sloughing off the scales (Figure 2A and 2D) and a large detachment of the skin and round ulcer were present (Figure 2B). Congestion and ulceration were noticed in gills from infected fish (Figure 2C). Lesions were in the external organs including skin, gills, and fins. The fish infected samples were taken from both cages and ponds from different location in north, centre, and south regions in Wasit province during the present study.

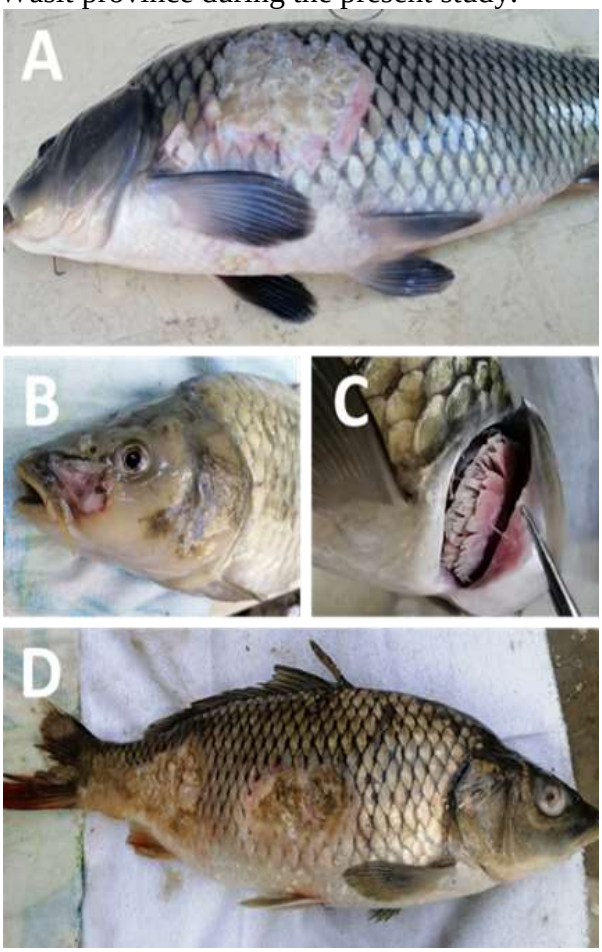

Figure 2. Swab taking area from infected fish including skin (A), (B) and (D), gills (C), and fins (D).

Fish are susceptible to wide variety of bacterial pathogens. Data in Table-1 revealed that the total bacterial isolates in fish samples from cage aquaculture were higher than pond culture system (56.7% and 37.5% respectively). As predicted in Figures 3, the rates of infection in cages were higher compared to ponds for the different regions of study encompassing, the centre, north, and south of Wasit throughout the entire period of the study, including September, October, November and December. Increasing the speed of infection in cage aquaculture system could be attributed to the greater probability of contact between reared and wild fish aggregated around cages' sites. Moreover, consistent with Lee et al. (14), other factors like agricultural and domestic sewages that are frequently discharged within the river may increase the organic contents of river's water and promote the expansion of varied species. Outbreaks of the infectious disease are usually caused changing in environmental state and stress, sudden fluctuation of temperature, crowding, low dissolved oxygen level, high ammonia level is the common factors related with citrobacteriosis (6). Several bacterial species identified during this study were pathogenic to fish, including *A. hydrophila*, *Edwardsiella tarda*, *P. aeruginosa*, *S. dysgalactiae* and *S. agalactiae* (15, 20, 25, 5, 11). Few species were reported harmful to humans like *Acinetobacter baumannii, Escherichia coli, M. morganii, S. aureus* and *Salmonella spp*. (18, 7, 17). Another is harmful to animals like *M. morganii, Salmonella spp.* and *Pasteurella spp*. (18, 4). Our findings are according to those obtained by Marcel et al. (16) who reported the isolation of various species of bacteria from one fish is related to the character of feed given to the fish, location of sampling sites, nearby human activities, and water quality at fish rearing area which may explain the increase rate of infection in fish harvested from cages compared to those from ponds.

Table 1. Total Prevalence rate (%) of bacterial isolates from different regions of Wasit province separated by sampling periods.

Sampling region	Culturing type	Percentage of positive bacterial culture			
		September	October	November	December
North	cage	50 %	70 %	70 %	30 %
	Pond	30 %	50 %	50 %	0 %
Canter	cage	70 %	80 %	80 %	40 %
	pond	50 %	60 %	60 %	20 %
South	cage	50 %	60 %	80 %	20 %
	pond	30 %	50 %	40 %	0 %
Average	cage	58 %			
	pond	37 %			

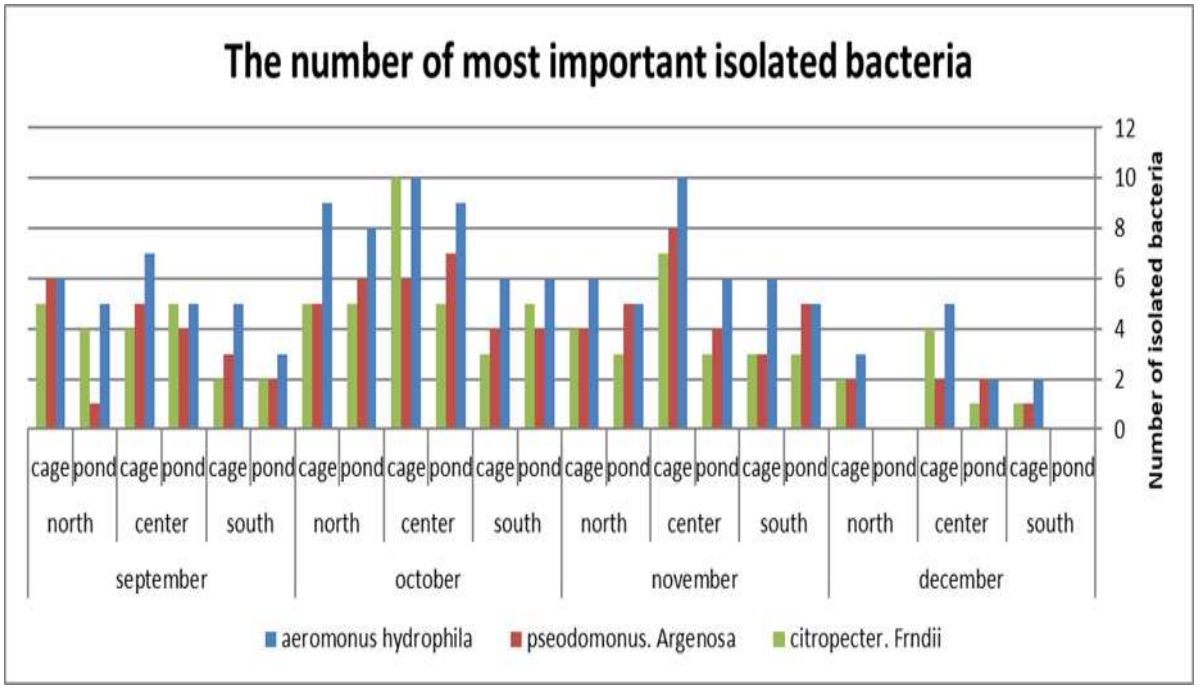

Figure 3. The prevalence of the most common bacterial pathogens involved in the development of ulcerative skin in cultivated fish during study period

In the present study, 15 different isolates from which three bacteria representing the most pathogenic bacteria affecting common carp fish were identified using morphological properties (Table 2), and series of biochemical tests and Gram staining (Table 3). Api® 20 E was used to confirm the results of culture-based techniques and biochemical tests**.** The isolated bacteria were *A. hydrophila*, *P. aeruginosa* and *C. freundii* as the most isolated bacteria and also *Enterobacter cloacae, Acinetobacter baumannii, Raoultella Terrigena, Vibrio cholera, E. Coli, Proteus Mirabilis, Proteus vulgaris, Klebsiella pneumoniae, S. aureus, Serratia liquefaciens, Salmonella Arizona and Edwardsiella tarda.*

Table 2. Colonies color of isolated bacteria on culture media

Type of bacteria	Color of colonies (culture media)
Aeromonas hydrophila	**Yellow (TCBS)**
Pseudomonas aeruginosa	**Translucent (Cetrimide agar)**
Citrobacter freundii	**Pink (MacConkey agar)**

Table 3. Biochemical characters of most important bacteria isolated from infected fish using API 20E system

		Aeromonus hydrophila	*Pseodomonus argenosa*	*Citropecter frndii*
Gram stain		**-**	**-**	**-**
Motility test		**+**	**+**	**+**
Catalase		**+**	**+**	**+**
Oxidase		**+**	**+**	**_-**
API 20E reactions: -				
ONPG	**β-galactosidase**	**+**	**-**	**+**
ADH	**Arginine dihydrolase**	**+**	**+**	**+**
LDC	**Lysine decarboxylase**	**+**	**-**	**-**
ODC	**Ornithine decarboxylase**	**+**	**-**	**+**
CIT	**Citrate utilisation**	**+**	**+**	**+**
H2S	**H2S production**	**-**	**-**	**+**
URE	**Urea hydrolysis**	**-**	**-**	**-**
TDA	**Tryptophan deamination**	**+**	**+**	**+**
IND	**Indol production**	**V**	**-**	**+**
VP	**Acetoin production**	**V**	**-**	**-**
GEL	**Gelatin hydrolysis**	**+**	**-**	**-**
GLU	**Glucose fermentation**	**+**	**+**	**+**
MAN	**Mannitol**	**V**	**V**	**V**
INO	**Inositol**	**V**	**V**	**V**
SOR	**Sorbitol**	**V**	**V**	**V**
RHA	**Rhamnose**	**V**	**V**	**V**
SAC	**Sucrose**	**V**	**V**	**V**
MEL	**Melibiose**	**V**	**V**	**V**
AMY	**Amygdalin**	**V**	**V**	**V**
ARA	**Arabinose**	**V**	**V**	**V**
Oxidase	**Cytochrome oxidase**	**+**	**+**	**-**

In general, the higher infection rates with *A. hydrophila*, *P. aeruginosa*, and *C. freundii* were detected in both cages and ponds rearing patterns (Table-4). In cages, the most detected pathogens were *A. hydrophila* (56.7%) as well as in *P. aeruginosa* (40.83%) and *C. freundii* (41.66%) and it is significantly ($P<0.05$) higher than the other isolated pathogens (Table-5). Among study ponds, the most detected pathogens were *A. hydrophila* (37.5%) and *P. aeruginosa* (35.8%), and *C. freundii* (25.8%) and it is significantly ($P\leq0.05$) higher than the other isolated pathogens (Table 6). *Aeromonas hydrophila* is survive and multiply in waters where there are high levels of organic matter and sewage (12), and normally present in the aquatic environment, especially in warm, organic-rich fresh water (22,3), these conditions are similar conditions where we collect or samples and may explain the higher percentage of *A. hydrophila* isolation. The present study results are in agreements with Omed and Alaa (2016) (1) were the find the most detected bacteria in causing skin lesions in north of Iraq (Sulaimani province) are *P. aeruginosa*, and *C. freundii*, however no *A. hydrophila* was detected because of low temperature conditions.

Table 4. Total results of pathogenic bacteria isolated from cages and ponds in Wasit and detected by Epi-20

Isolates	Prevalence (%)		x^2	Interpretation
	Cage	Pond		
Aeromonas hydrophila	**56.7**	**37.5**	**3.946**	**S**
Pseudomonas aeruginosa	**40.83**	**35.8**	**2.623**	**NS**
Citrobacter freundii	**41.66**	**25.8**	**4.058**	**S**

x^2: Chi-square, S: Significant, NS: Non-significant

Table 5. The percentage relative abundance of bacterial communities detected by Epi-20 in cage rearing system

Isolate	Total no.	Prevalence		P-value
		No.	%	
Aeromonas hydrophila	120	68	56.7 **	
Pseudomonas aeruginosa	120	49	40.83 *	0.015
Citrobacter freundii	120	50	41.66 *	

Table 6. The percentage relative abundance of bacterial communities detected by Epi-20 in pond rearing system

Isolate	Total no.	Prevalence		P-value
		No.	%	
Aeromonas hydrophila	120	45	37.5 **	
Pseudomonas aeruginosa	120	43	35.8 **	0.05
Citrobacter freundii	120	31	25.8 *	

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