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

المستخلص

أجريت هذه الدراسة للكشف عن العوامل المسببة للأمراض والتي تسبب الأعضاء الخارجية لأسماك الكارب في محافظة واسط في كارب Cyprinus carpio، والتي تم إجراؤها من ثلاث مناطق مختلفة. تم إجراء الدراسة في البيئات المختبرية في جامعة بغداد ومركز الصحة العامة في بغداد من شهر أيلول إلى كانون الأول 2020. في الدراسة الحالية تم استخدام 240 من أسماك الكارب Cyprinus carpio L. من ثلاثة مناطق تمثل الشمال، وسط، وجنوب المحافظة. وأخذت عينات من قرح الجلد من المناطق المصابة في جسم السمكة (الجلد، الخياشيم، والزعانف) ونقلها إلى المختبر لإجراء الاختبارات المخبرية. نشرت النتائج أن أنواع البكتيريا الرئيسية المسببة للأمراض المرتبطة بتقرح الجلد والخياشيم والزعانف هي أ. هيدروفيل، Pseudomonas aeruginosa، Citrobacter freundii. كانت معدل الإصابة أعلى في الأقفاص مقارنة بالاحواض بسبب زيادة التلوث بمياه الصرف الصحي والمركبات العضوية والضغوطات المادية التي تزيد من درجة الإجهاد. أظهرت الدراسة أن زيادة في عدد الأسماك في الأحواض مثل Pseudomonas aeruginosa وبكتيريا Citrobacter freundii، تزيد من معدل الإصابة بالمرض بسبب التلوث العضوي.
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 province. 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 september, 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 37°C. Then MacConkey agar, SS agar, mantoil salt agar were streaked by loopful from the broth and incubated at 37°C for 18-24 hours. pink colonies, lactose ferment were transfer to TCBS agar and Blood medium and incubated at 37°C 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 37°C 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 37°C 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 37°C, 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 37°C, then slants were kept at 4°C for a few weeks. For long storage, the pure isolated bacterium was inoculated in BHIB containing 15% glycerol and incubated for 24 hours at 37°C, 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.

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 baumanii, *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

| Character                  | Aeromonas hydrophila | Pseudomonas argenosa | Citrobacter freundii |
|----------------------------|----------------------|----------------------|----------------------|
| Gram stain                 | -                    | -                    | -                    |
| Motility test              | +                    | +                    | +                    |
| Catalase                   | +                    | +                    | +                    |
| Oxidase                    | +                    | +                    | -                    |
| API 20E reaction           |                      |                      |                      |
| ONPG                       | β-galactosidase      | +                    | -                    |
| ADH                        | Arginine dihydrolase | +                    | +                    |
| LDC                        | Lysine decarboxylase | +                    | -                    |
| ODC                        | Ornithine            | +                    | +                    |
| CIT                        | Citrate utilisation  | +                    | +                    |
| H2S                        | H2S production       | -                    | -                    |
| URE                        | Urea hydrolysis      | -                    | -                    |
| TDA                        | Tryptophan deamination | +                    | +                    |
| IND                        | Indol production     | V                    | -                    |
| VP                         | Acetoin production   | V                    | V                    |
| GEL                        | Gelatin hydrolysis   | V                    | V                    |
| GLU                        | Glucose fermentation | V                    | V                    |
| MAN                        | Mannitol             | V                    | V                    |
| INO                        | Inositol             | V                    | V                    |
| SOR                        | Sorbitol             | V                    | V                    |
| RHA                        | Rhamnose             | V                    | V                    |
| SAC                        | Sucrose              | V                    | V                    |
| MEL                        | Melibiose            | V                    | V                    |
| AMY                        | Amygdalin            | V                    | V                    |
| ARA                        | Arabinose            | 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 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≤0.05) 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 (%) |
|-------------------|----------------|
|                   | Cage | Pond | $x^2$ | Interpretation |
| 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 No. | Prevalence % | P-value |
|-----------------------|-----------|----------------|-------------|---------|
| Aeromonas hydrophila  | 120       | 68             | 56.7 **     | 0.015   |
| Pseudomonas aeruginosa| 120       | 49             | 40.83 *     |         |
| 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 No. | Prevalence % | P-value |
|-----------------------|-----------|----------------|-------------|---------|
| Aeromonas hydrophila  | 120       | 45             | 37.5 **     | 0.05    |
| Pseudomonas aeruginosa| 120       | 43             | 35.8 **     |         |
| Citrobacter freundii  | 120       | 31             | 25.8 *      |         |

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