Wasit Journal for Pure Science

Journal Homepage: https://wjps.uowasit.edu.iq/index.php/wjps/index
e-ISSN: 2790-5241 p-ISSN: 2790-5233

Biochemical and Molecular Detection of *Aeromonas Hydrophila* Isolated from Infected *Cyprinus Carpio* in Salah Al-Din Governorate

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DOI: https://doi.org/10.31185/wjps.503
Received 20 July 2024; Accepted 29 August 2024; Available online 30 September 2024

ABSTRACT: This study aimed to identify the presence of *Aeromonas hydrophila* bacteria in diseased common carp (*Cyprinus carpio*) fish collected from Salah al-Din Governorate in Iraq. For this purpose, 128 swabs were obtained from skin, gill and fin lesions of 70 fish showing symptoms of hemorrhagic sepsis, skin ulcers and boils, and fin and gill rot. Through different biochemical tests, the isolates showed negative results for Gram stain, methyl red, lactose fermentation, H_2S, and string test, while positive results were recorded for glucose fermentation, oxidase, motility test, catalase, Voges-Proskauer, indole, citrate utilization, and gelatin hydrolysis. Eleven isolates were confirmed as *A. hydrophila* by amplification of the hemolysin gene fragment (hlyA) by conventional PCR. This study represents the first comprehensive study to characterize *A. hydrophila* bacteria from infected common carp fish in Salah al-Din Governorate. The findings of this study provide valuable insights for developing genetic diagnostic strategies for *A. hydrophila* infections in this region.

Keywords: *Aeromonas hydrophila*, Cyprinus carpio, String Test, Hemolysin (hlyA).

1. INTRODUCTION

Aquaculture is the practice of farming aquatic organisms such as fish, mollusks, crabs, and aquatic plants. Aquaculture production especially pertains to the yield of aquaculture endeavors, with the purpose of being ultimately harvested for food (1). According to the FAO's report on Iraq's fisheries and aquaculture sector this country is ranked fifty-third in global aquaculture production out of 177 countries. The values of the aquaculture production index (in metric tons) in Iraq reached 25,737.00 in 2018. Over the past 58 years, it reached a maximum value of 31,814.00 in 2017 and a minimum value of 0.00 in 1960(2). Common aquaculture in Iraq is mainly associated with Carp farming (3). The common carp species, *Cyprinus carpio*, was initially brought to Iraq in 1955. Subsequently, a distinct variety of the species was introduced in 1982 from Hungary. In 2009, Enmaa Agricultural Enterprises bought a fresh consignment of common carp from Hungary for production purposes (4). Carp is an important species in aquaculture in Iraq due to its high growth, annual production, and high yields(5). Diseases have an ever-greater impact on fish in the global expansion and intensification of the aquaculture system(6). In a study reported by Faruk et al (2004)

they indicated financial losses amounting to approximately 15% due to diseases affecting fish (7) . The World Bank (2014) reported that the diseases have an estimated economic impact of US$6 billion annually on the global aquaculture industry(8). Bacterial diseases are the most common infectious issue in commercial fish farming and aquaculture. Infectious bacterial diseases pose the most common risk in trading Fish farms and ornamental fish. Some of bacteria are common responsible of infectious diseases in farmed fishes and ornamental fishes. Aeromonas spp., a prominent bacterial pathogen, causes a range of ailments including kidney disease, ascites, fish dropsy, intestinal flushing, motile aeromonad septicemia , tail, mouth fungus, and fin rot, and spinal diseases (9). *Aeromonas hydrophila* is the causative agent of MAS (motile Aeromonas septicemia) the disease is characterized by abdominal ascites and redness of the mouth, Hemorrhagic patches in the outer surface and surroundings of the anus(10). It is regarded as an opportunistic infection that only causes disease in fish when they are experiencing stress. *Aeromonas hydrophila* was commonly found in different fish lesions associated with epizootic ulcerative syndrome (EUS) (11). Carp exhibits a high degree of tolerance towards unfavorable environmental circumstances, including low levels of dissolved oxygen, pH, and variations in turbidity (12). At now, in the Salah Al-Din Governorate, the occurrence of diseases is a significant issue that is limiting the practice of carp farming. Nevertheless, as a result of insufficient diagnostic assistance, farmers are experiencing significant economic losses. There is a persistent dearth of information regarding the pathogen linked to the diseases affecting farmed carp in the region.

Therefore, the objectives of the current study to isolate *A. hydrophila* from skins and gills in Infected *Cyprinus carpio* who have symptoms of Aeromonasis and characterization of isolates by biochemical tests and genetic detection of the pathogen Hemolysin (hlyA) fragment.

2.MATERIALS AND METHODS

2.1 Field investigation and specimen collection:

Carp fish that showing clinical signs of the disease were obtained from four earthen fish ponds, especially in the Dujail district, and Tikrit city. The average size of each ponds is approximately 26,909,787 feet and a depth of 2-3 feet, with a good water treatment and water exchange system. The farm owner stocked 6 inches of 70-day-old *Carp juveniles* at a density of approximately 4,500 per pound. A total of 128 swabs were collected from 70 infected carp fish over a period of 8 months, from the beginning of July 2023 until the end of February 2024. A visual examination was conducted to confirm the presence of clear signs and symptoms of Aeromonasis on the fish. These included skin ulcers, swelling of the abdominal area, bulging eyes, and bleeding spots around the anus opening (Fig1). The swabs stored in tubes containing peptone water were then transferred to sterile containers and kept cool until they reached the laboratory, where they were cultured.

2.2 Isolation of pathogenic bacterial isolates

After incubating the swabs for 24 h at a temperature of 28 °C, they proceeded to culture them directly on MacConkey agar (M.A) (Himedia, India), and incubated them at a temperature of 30 °C for 24 hr. Subsequently, the well-isolated and non-fermenting lactose (LN) colonies (Fig 2) were cultured on Aeromonas Agar Base (A.A.B) (Dinkelberg Analytics, German), with incubation temperatures of 30°C for 24-72 h, in order to facilitate pure colony growth and preparation for phenotypic and biochemical tests. All media in this study were prepared according to their manufactured instructions.

Primary characterization and identification of isolates The Distinguishing features of Aeromonas colonies were determined by examining their morphological characteristics, including shape, color, surface texture, odor and, transparency on Aeromonas agar base medium (Fig 3), as well as the wavy phenomenon and the hemolysis pattern on modified blood medium (Fig 4) which was prepared according to Winn Washington et al., 2006 method, and lactose non fermentation on MacConkey agar medium (13).

2.2.1 Gram's staining

Bacterial cells from pure colonies were stained with Gram's dye(aTom, England) and examined under microscope(Fig 5) at ×100 magnification using an oil lens(14).

2.2.2 Motility test

The motility test was performed using the hanging drop technique, with the bacteria cultivated in both semi solid nutrient agar and broth media. Initially, a drop of bacterial inoculated nutrient broth was meticulously put onto a glass slide. Subsequently, the slide was delicately turned upside down to enable the culture drop to suspend. Ultimately, the descent was detected using a light microscope.(15)**.** In order to demonstrate the phenomenon of motility, freshly grown bacterial colonies were inoculated with a sterile stabbed needle in the center of a semi-solid agar plate (Fig 6). Then the dishes were incubated by face up for 24-48 h at 30°C, with the aim of observing the migration of bacteria through the agar from the center of the dish towards the periphery(16).

2.2.3 Oxidase test

An oxidase test was carried out with the assistance of oxidase detection strips (Oxoid, BIOANALYSE TIBBI MALZ., Ankara, Turkey). In a nutshell, a little amount of bacteria was placed on the strips that contained oxidase reagents (N, N-dimethyl-1,4-phenylene diammonium chloride), and the color change was observed after ten seconds had passed (Fig 7).

2.2.4 Catalase test

The test was conducted on some colonies grown on nutrient agar (N.A.) (Himedia, India).The colonies were taken by using a loop, placing them on the surface of a sterile glass slide, then adding a few drops of a 3% hydrogen peroxide solution (Fig 8). This method was selected as it has been observed that the production of gas bubbles from bacterial growth within several seconds indicates a positive reaction to the test(17).

2.2.5 Sugar Fermentation and Gas Production Test

The suspected Aeromonas species isolates should be incubated at 30°C for 22 h in a slanted tube containing the triple sugar iron (Fig 9) medium and the positive colonies of the oxidase test mentioned above. The fermentation reaction on the surface medium will exhibit a yellow acidic(A) butt, a red alkaline(K) slant surface, and varying H_2S formation(18).

2.2.6 IMViC Test:

I. Indole production Test

Once the peptone water has been prepared and distributed into tightly-closed test tubes, it should be cooled and inoculated under sterile conditions using a portion of a purified young colony, taken at 18 to 24 h of age. The tubes were incubated at 30 °C for 24-28 h, after which 0.5 ml of Kovacs reagent is added to each tubes and shaken well. The appearance of a cherry red ring (Fig.10) on the surface of the broth within a few minutes of adding the reagent is indicative of the bacteria's capacity to decompose tryptophan and produce indole, thereby yielding a positive result(19).

II. Methyl red test (MRT)

MR-VP Medium has been prepared, a portion of a pure young colony, approximately 18-24 h Cells from pure colonies were stained with Cram's dye and examined under a standard microscope at ×100 magnification using an oil lens, is inoculated into the broth and incubated at 30 °C for 24-48 h. The red methyl indicator is then added (Fig 11). Upon the medium's color transitioning to red following reagent addition, the result is recorded as positive. In the event that the yellow color of the medium persists even after the addition of several drops of the reagent, this indicates that the test result is negative(20).

III. Voges-Proskauer Test

This test was conducted by using MR-VP broth inoculated with the purified colony of bacteria *A. hydrophila* One milliliter of Vp1 solution was added, followed by one milliliter of Vp2 solution (Fig 11), and the mixture was incubated at 30 °C for 45 to 60 minutes. The result was then read, and it was noted that a positive result was indicated by a color change in the medium from yellow to shades of red. In the event of a negative result, namely, a failure to observe a color change from yellow, the medium will remain unaltered(21).

IV. Citrate utilization test

This test was conducted by inoculating an agar-Simmons citrate medium on the slant surface by touching the tip of a needle to a freshly cultured colony (Fig 12). The inoculated medium was then incubated at 30°C for 18 to 24 h. The growth of the organism was visible on the slanted surface, and the medium will change color to blue(22).

2.2.7 String test

String test is a key diagnostic tool for differentiating between the genus Vibrio spp, and Aeromonas spp. genus. This is achieved by transferring several recently developed bacterial colonies to slide and adding one to two drops of a 5% sodium deoxycholate solution and mixing for several minutes. The loop is then lifted to observe the result. When the suspension loses its turbidity and produces a sticky thread, this indicates a positive result. However, when the mixture remains turbid, this indicates a negative result (Fig 13). This means that no sticky thread is formed(23).

2.2.8 Gelatin hydrolysis test:

The isolated colony of *Aeromonas hydrophila* was transferred from a nutrient agar plate to a gelatinous nutrient medium in sealed laboratory tubes by using a loop. The tubes were incubated at

30°C for approximately three days. After observing bacterial growth in the medium, the tubes were transferred and incubated in a refrigerator at 4 °C for 25 minutes. The results were checked by observing whether the medium had solidified or not and comparing it to tubes of the same medium that not inoculation with bacteria. It should be noted that the Aeromonas spp. can liquefy the gel (Fig 14) (24).

2.3 Genomic identification of *A. hydrophila*

The isolated colonies were stained with Gram stain and examined under the microscope under oil immersion to check for purity. The DNA was then extracted from pure isolates subsequently and polymerase chain reaction (PCR) amplification of a Hemolysin(hlyA) fragment was run.

2.3.1 DNA Extraction and Purification

The DNA extraction was conducted using the GenElute™ Bacterial Genomic DNA Kit(25). Chromosomal DNA screening was performed on all clinical isolates following the directions provided by the manufacturer. The total DNA was utilized for the detection of hlyA gene. Table 1 shown the DNA primers (hlyA) F, R used in present study.

Table 1. primer used for the amplification of hlyA gene of *Aeromonas hydrophila*

Gene	primers	DNA sequences	Product size(bp)	Reference
hlyA	F	GGC CGG TGG CCC GAA GAT GCA GG	597	(21)
	R	GGC GGC GCC GGA CGA GAC GGG		

2.3.2 PCR thermocycling conditions and agarose gel electrophoresis

A polymerase chain reaction (PCR) was performed using primers specific to the hlyA gene in A. hydrophila. The PCR mixture had a total volume of 25.00 µl, with 2.00 µl of template DNA and 12.50 µl of a master mix. The master mix consisted of all the deoxy nucleoside triphosphates, MgCl2, and Taq DNA polymerase. In addition, the assay utilized 1.00 µl of a 10.00 A concentration of pM per µl of each primer, along with 8.50 µl of sterile double-distilled water. The thermal profile consisted of an initial step at 94 °C for 5 minutes (one cycle), followed by 30 cycles involving denaturation at 94 °C for 30 seconds, annealing at 68 °C for 30 seconds, and extension at 72 °C for 2 minutes. The reaction was considered negative when the negative controls were used, in which the DNA preparation was replaced with PCR water. On the other hand, positive controls were used to verify the precision of the reaction. Afterwards, the PCR products were analyzed using electrophoresis on a 1.5% agarose gel with safe view and observed using UV trans-illumination. If a 597 base pair band was detected for the *hlyA* gene, the isolate was identified as *A. hydrophila*.

3. RESULTS AND DISCUSSION

This study aims to isolate and identify *A. hydrophila* using biochemical and PCR techniques from infected carp fish. 128 samples from 70 fish were collected which showing clinical signs of infection. While 56 isolates were classified as Aeromonas spp. based on colonies phenotyping

on Aeromonas agar base media. Among them, 11 isolates were classified and identified as *A.hydrophila* according to biochemical test results (Table 2) and PCR analysis (Fig.15).

Table 2. Biochemical identification of *Aeromonas hydrophila*. Dates are percentage(n=56)

Biochemical Tests	***A.hydrophila***(22)	**Positive**	**Negative**
Gram stain	-	-	100
Motility	+	57.89	31.58
Oxidase	+	28.57	71.43
Catalase	+	100	-
Indole	+	35.71	64.29
H2s	-	-	100
Simmon's citrate	+	89.3	10.7
Voges-Proskauer	+	50	50
Lactose	-	0	100
Glucose	+	67.9	32.1
Gelatinase	+	62.5	37.5
String	-	80.36	19.64

Aeromonas hydrophila is a ubiquitous pathogenic bacterium that is especially common in aquaculture and leads to substantial economic losses in this industry. The isolation, identification, and confirmation of microorganisms that cause diseases in fish are essential for accurately diagnosing suspected illnesses. A common bacterium found in cultured organisms is *A. hydrophila*.(28). *A. hydrophila* is an opportunistic bacterium that may cause extensive damage and significant losses to fish breeders. In a recent study, samples of 70 infected fish with Aeromonasis clinical signs were purified.

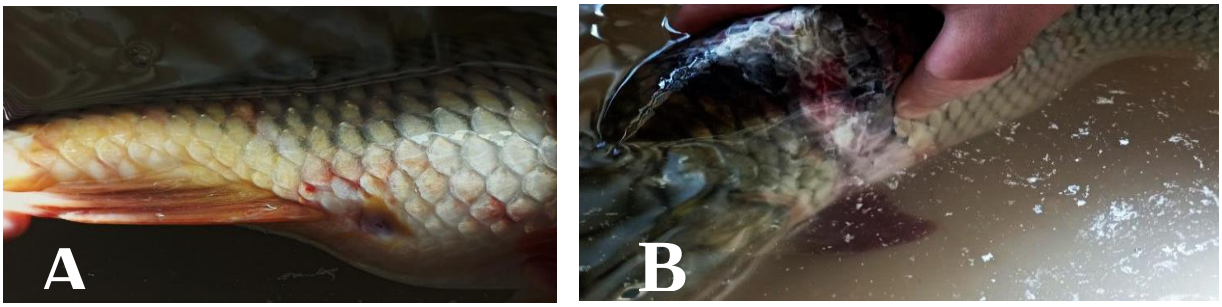

Figure1: showing symptoms of Aeromonasis: (A) Bleeding spots around the anus opening and abdominal swelling; (B)Skin ulcers

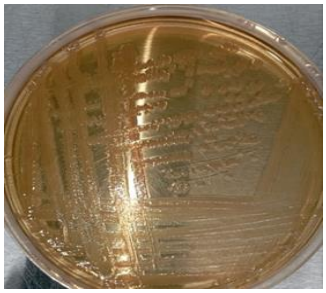

Figure 2: Lactose nonfermention, *A.hydrophila* on MacConkey agar media

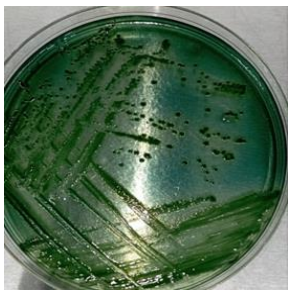

Figure 3: *A.hydrophila* on aeromonas agar base media

Figure 4; Beta hemolysis, *A.hydrophila* on 7%blood agar media

Figure 5: Gram negative rods

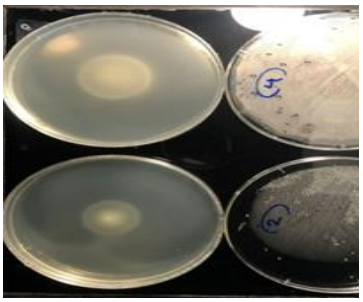

Figure 6: positive Motility test

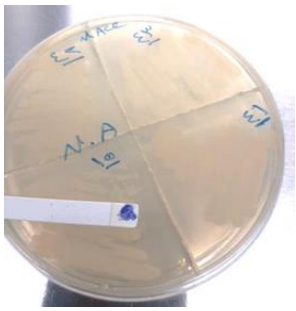

Figure 7: Positive oxidase

Figure 8: Positive catalase

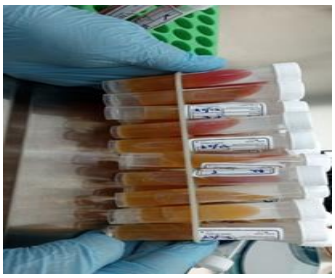

Figure 9: K/A, A/A, TSI agar media

Figure10: Positive indole ring test

Figure11: MR-VP test

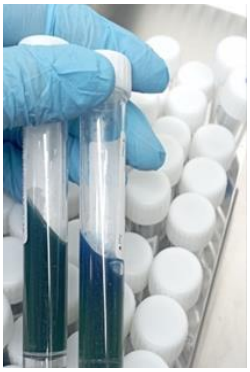

Figure12: Citrate utilization test on Simmon's citrate media agar

Figure13: Negative String test

Figure14: Gelatin hydrolysis test

A total of 128 fish swabs were examined in the laboratory, resulting in the identification of 56 colonies of gram-negative bacteria. These bacteria were classified as either motile or non-motile *Aaeromonads* species based on their particular morphological, physiological, and biochemical properties. Out of the 70 fish that were sampled and found to be contaminated, 15.7% of them were identified as *A. hydrophila*. In the end, a total of 11 isolates were determined to be *A. hydrophila* (Fig.16). The ability to diagnose and establish phylogenetic links within the species enables the mitigation of hazards and disease control (28–30).

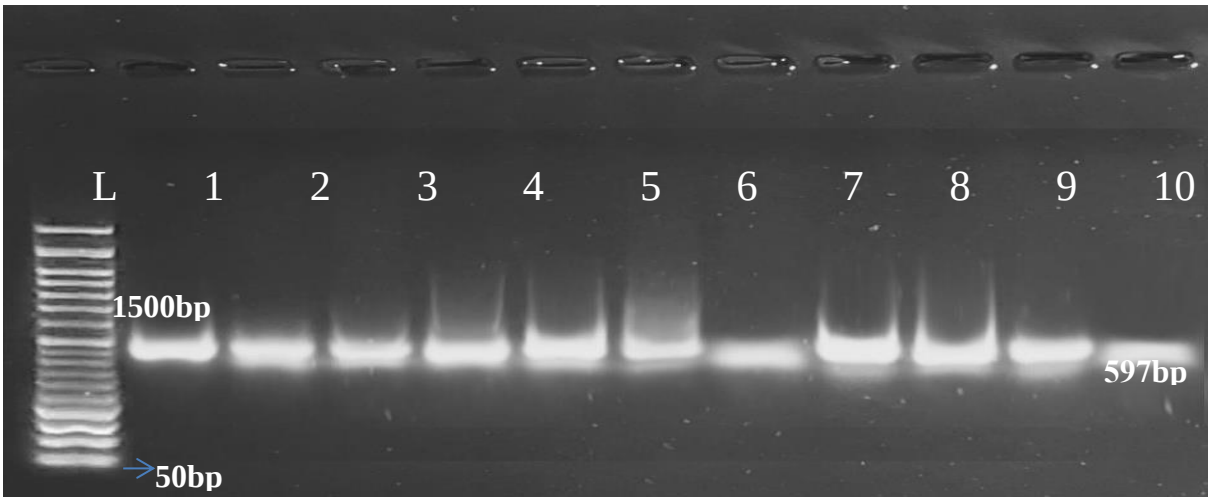

Figure15: identified as *A. hydrophila* using PCR analysis (*hlyA*)

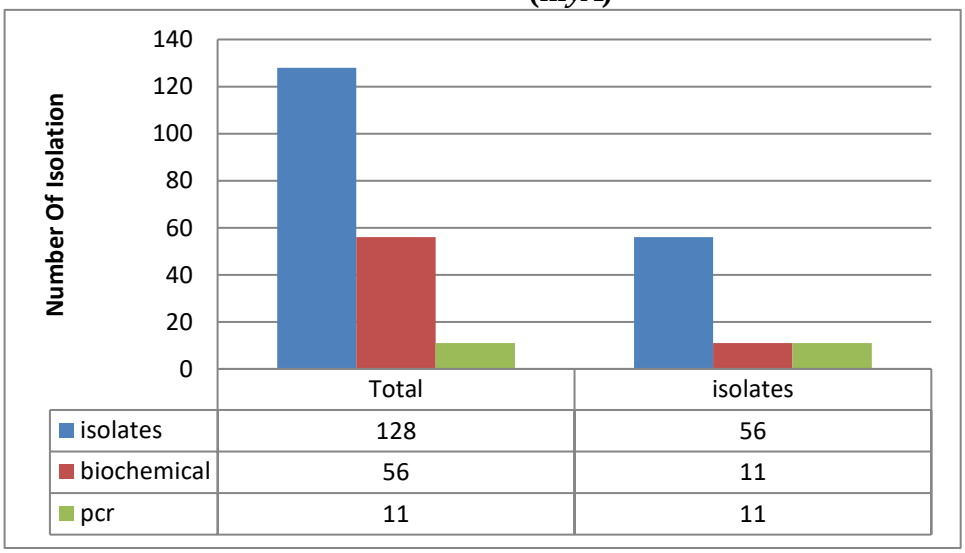

Figure 16: Numbers of *Aeromonas hydrophila* isolates characterised by biochemical test and PCR.

The identification of Aeromonas species at the species level can be effectively achieved using PCR, making it a valuable technique for detecting outbreaks of Aeromonas species. In addition, the *hlyA* gene can be utilized for molecular diagnostics of the fish pathogen. This technology has been proven to be highly reliable and enables rapid identification and determination of bacterial

species in the aquaculture business. The process of isolating and identifying *A. hydrophila* from fish ponds involved using PCR assay with hlyA primers to amplify the *hlyA* gene fragment. The most efficient approach for identifying virulence factors specific to the *Aeromonas* genus is by detecting the presence of *hemolysin* genes (*hylA*) and *aerolysin* (*aerA*) genes (31).

4. CONCLUSION

Aeromonas hydrophila is a widely distributed pathogenic bacteria, especially common in warm-water aquaculture, that leads to substantial financial losses in the aquaculture sector. We have successfully provided the first evidence of isolating, identifying, and characterizing *A. hydrophila*. This is the process of identifying and describing *A. hydrophila* in ill juvenile common carp fish in Salah-Adin Governorate. Our hypothesis is that this bacterium could be one of the primary pathogens in common carp aquaculture in Iraq. Our current findings provide a new horizon for the development of diagnostic strategies for *A. hydrophila*. However, further studies are necessary to ascertain the full molecular, serological, and serological characteristics of the organism. Furthermore, the identification of *A. hydrophila's* virulence factors, specifically in common carp, also requires further investigation.

5. ACKNOWLEDGEMENT

I would like to convey my sincerest gratitude to Dr. Median Nasser Ali for his unwavering collaboration and support in the collection of samples throughout the preparation of this research. I would also like to extend my appreciation to my colleagues in the laboratory department of the veterinary hospital in Nineveh Governorate for their assistance in data collection and analysis.

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