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Isolation, molecular identification and resistant profile of *Aeromonas hydrophila* from chicken meat

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ABSTRACT

Aeromonas hydrophila have recently received great attention due to their link with serious diseases in humans if transmitted through food, as well as their exceptional ability to withstand harsh environmental conditions. The study aimed to investigate the presence of *A. hydrophila* in chicken meat and determine their potential resistance to antibiotics. One hundred and twenty-one samples of chicken meat were collected from Dohuk, Erbil, and Sulaymaniyah, cities in northern Iraq. Traditional methods were used, including culture and biochemical tests, then molecular confirmation by amplifying the *16S rRNA* gene, and then all isolates were subjected to antibiotic susceptibility testing. The results showed that 30/121 (24.79%) of the samples were contaminated with *A. hydrophila*. The results of the antibiotic resistant profile revealed that (90%) of the isolates were resistant to cephalothin, and (83.33%) were resistant to amoxicillin and tetracycline. Most of the isolates also showed sensitivity to ceftriaxone, cefixime, nitrofurantoin, ciprofloxacin, chloramphenicol and trimethoprim in different proportions. The results of this study indicate that the presence of *A. hydrophila* in poultry meat samples poses a major health risk and may cause foodborne diseases, thus increasing the possibility of causing foodborne diseases. Therefore, it is important to pay more attention to these bacteria, and strict hygienic practices must be adopted to reduce bacterial contamination.

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Introduction

Poultry meat is considered one of the widely consumed meats by human globally, because it is a good source of proteins, convenient with low prices and contains less fat compared to red meat (Barbut & Leishman 2022). *Aeromonas hydrophila* is a widely existing Gram-negative bacillus, non-spore forming, mesophilic bacteria, positive for oxidase- and catalase, motile by means of polar flagella, belonging to Aeromonadaceae family (Enany et al. 2015). *A. hydrophila* tolerates a broad range of temperatures from 4°C to 45°C with inconstant growth in a pH ranging from 5 to 9. These bacteria also have the ability to produce different types of toxins and form biofilms (Sekavec et al. 2013).

Aeromonas spp. have received a great attention because some strains of these microorganisms have been linked to serious illnesses in human when transmitted through food and water (Fernandez-Bravo & Figueras, 2020). *A. hydrophila* strains are important emerging foodborne pathogens because they have the ability to grow at low temperatures as well as their exceptional ability to tolerate extreme conditions in the environment (Daskalov

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2006). *A. hydrophila* has been linked to 85% of gastroenteritis in human (Duman et al. 2018).

Aeromoniasis is globally distributed and the disease in poultry has been reported in many parts of the world causing localized or systemic infections either alone or in combination with other infections (Abd El-Ghany 2023). Since *Aeromonas* spp. grow easily at refrigerator temperatures, the pathogen has been isolated from a variety of foods of animal origin including poultry meat (Kumar et.al. 2000).

A. hydrophila is among the emerging pathogens involved in food borne diseases causing a serious risk to people safety and health. Young children, aged people, and individuals with flawed immune systems are all reported to be at higher risk of *Aeromonas* spp. infection (Tsheten et al. 2016), it is also cause a variety of infections in humans, such as gastroenteritis, septicemia, skin necrotizing fasciitis, gangrene, meningitis, peritonitis, and hepatobiliary infections (Figueras & Beaz-Hidalgo 2014; Jwher, et al., 2021).

Aeromonas infections in poultry has been documented in different parts of the world with disconcerting effects, thus isolation of *A. hydrophila* from chicken meat represent a public health concern, therefore, it is crucial to regularly and periodically test flocks of chickens in various geographic locations, and it is very important to educate the public about the risks associated with *Aeromonas* infection (Praveen et al. 2016; Ezzulddin et al., 2020).

Given the growing concerns about *Aeromonas hydrophila* and its ability to withstand adverse environmental conditions, this study raises serious questions about the possibility of this taxon being transmitted through poultry meat, especially antibiotic-resistant strains, to humans, causing different diseases.

Currently, there is limited data investigating *A. hydrophila* in poultry meat in the study area in Iraq. Therefore, the present work is performed to investigate the prevalence of *A. hydrophila* in chicken meat samples obtained from poultry slaughterhouse and retail markets, and to determine their antibiotic resistance profile.

Materials and Methods

Sampling

A total of 121 raw local poultry meat samples were included in this study. Samples were collected for the period from March to October 2022. Meat samples were collected aseptically using a sterile plastic bags, labeled and placed in an ice box then transported immediately to the Research Center in the College of Veterinary Medicine/ University of Duhok for diagnostic analysis.

Bacterial Isolation

Twenty-five grams from each sample were minced, homogenized with 200 ml of alkaline peptone water, and incubated overnight at 37 °C an enrichment step. A loopful of the enriched sample was inoculated on blood agar supplemented with 5% defibrinated sheep blood, then incubated at 37°C for 24 hrs. Beta-hemolytic (2-3 mm) colonies were directly subcultured onto MacConkey agar and as previously described (Taha et al. 2021).

Biochemical analysis using the "VITEK 2" system

VITEK® 2 Gram-Negative Identification Cards (bioMerieux Inc, France) were used to identify the isolates. The technique was performed following the manufacturer's instructions and the resulted data was compared to the respective database in the computer attached to the VITEK-2 operator system. The desired organisms were matched with the strains used as references and stored in the system software (Elbehiry et al. 2019).

Molecular Confirmation

For final confirmation of the isolates as A. hydrophila, the 16S rRNA gene was amplified by conventional polymerase chain reaction (PCR).

DNA extraction

Thermal extraction (the boiling method) is used for genomic DNA extraction. According to Taha & Yassin (2019), (3-5) morphologically similar colonies were selected and added to a 1.5 sterile tube filled with 300 µl of sterile double distilled water, vortexed for 30 seconds and then heated at 95ºC for 10 min using Stuart Dry Block Heater (Thermo Fisher Scientific™ USA). The tubes were cooled immediately with ice and after that centrifuged for 10 min. About 150 µl of supernatant was transferred to another sterile tube and used as a template DNA for PCR. The extracted DNA samples were stored at -20 °C till used for further investigations by polymerase chain reaction (PCR).

Conventional PCR

For confirmation of the isolates as *A. hydrophila*, *16S rRNA* universal gene amplified, the primer set listed in (Table 1) was used. The amount of PCR mixture was 25 µL which contained 1 µL forward primer, 1 µL reverse primer (Table 1), 12.5 µL AddStart Taq Master (ADDBIO INC, Korea), 5.5 µL sterilized double distilled water, and 5 µL DNA template.

Table 1 Primers used for amplification of *16S rRNA* gene / *A. hydrophila.*

Target Gene	Primer sequence (5′-3′)	Amplicon size (bp)	Reference
16S rRNA	**F**- AGAGTTTGATCCTGGCTCAG **R**- GGTTCACTTGTTACGACTT	1498	(Abdulhasan et al. 2019)

The DNA samples were amplified using Thermal-cycler program (Applied Biosystems GeneAmp® PCR System 9700) which was adjusted according to Abdulhasan et al. 2019 with some optimizations as follows: Initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 45 seconds then annealing at 56°C for 1min., followed by extension at 72°C for 1min., and a final extension at 72°C for 7 minutes.

After completion of all cycles, the products were run on 1.5% agarose gel stained with safe dye (ADDBIO INC, Korea) and observed under UV Transilluminator (Vilber Lourmat Super Bright-France). To find the size of the PCR product the (100 bp) DNA ladder H3 RTU (GeneDireX, Taiwan) was used.

Sequencing identity analysis

For confirmation of prospective *A. hydrophila* isolates, partial *16S rRNA* gene sequencing was employed. PCR amplicons of the studied species were sent to Immunogene Center / North Korea for DNA sequencing, the outcomes were submitted to the similarity search using Basic Local Alignment Search Tool (BLAST) program at the "National Center for Biotechnology Information" (NCBI) (Navarro & Martínez-Murcia 2018).

Antibiotic Susceptibility testing

The test was performed according to the Clinical and Laboratory Standards Institute guidelines using Kirby-Bauer disc diffusion method on Mueller-Hinton agar (CLSI 2020). Few colonies of the fresh isolates were selected and suspended in brain-heart infusion broth. Muller-Hinton agar plates were inoculated by streaking across the whole agar surface more than three times. Discs saturated with antimicrobial agents were placed onto the agar surface, then incubated at 37 °C for 24hrs. According to the CLSI, the inhibition zone was measured, and the test results were divided into three categories: resistant, intermediate and sensitive. Twelve types of antibiotics (Bioanalyse®/Turkey) include: Tetracycline (TE 10 µg), Cephalothin(KF 30 µg), Amoxicillin(AX 10 µg), Ciprofloxacin(CIP 10 µg), Cefixime(CFM 5 µg), Streptomycin(S 5 µg), Ceftriaxone(CRO 10 µg), Erythromycin(E 10 µg), Gentamycin(CN 10 µg), Trimethoprim(TMP 10 µg), Nitrofurantoin(F 100 µg) and Chloramphenicol(C 10 µg) were included for this testing.

Results

The results of conventional cultural methods revealed that out of 121 chicken meat samples, 42 (34.7%) of the samples were positive for motile *Aeromonas* species. This result was obtained depending on the hemolytic activity of the bacteria when a zone of hemolysis around the colonies was observed on blood agar plates and pale colonies appeared on MacConkey agar indicating that the isolates are non-lactose fermenters (Figure 1).

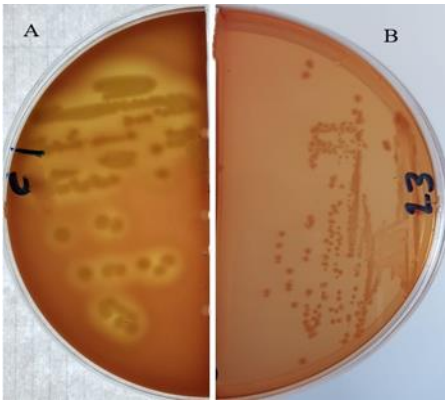

Fig 1. Phenotypic characterization of *Aeromonas* isolates; **A**- β-hemolytic colonies on blood agar. **B**- Pale- non lactose fermenting colonies on MacConkey agar.

Forty-two morphologically identified isolates were subjected to the VITEK 2 test, and the results showed that (34) isolates were identified to be *A. hydrophila;* (6) isolates were other species of the genus *Aeromonas* and two isolates were unverified by this technique (Figure 2).

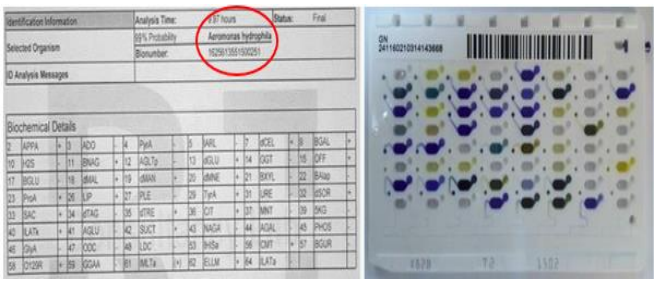

Fig 2. VITEK-2 system result for *A. hydrophila* isolates.

Molecular confirmation

The final confirmation for the isolates that have been identified as *A. hydrophila* by Vitek-2 system was done by amplification of the *16S rRNA* gene. DNAs from the isolates were extracted and the *16S rRNA* gene amplified using PCR; the amplified DNA showed a product size of 1498 bp (Figure 3).

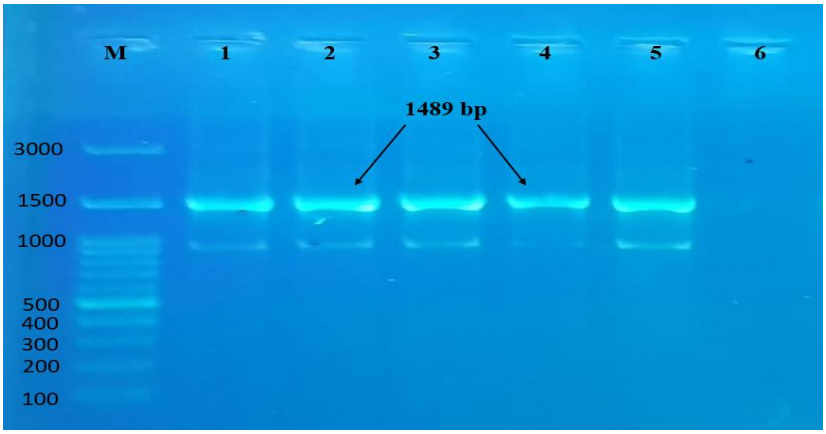

Fig 3. PCR amplification for the *16S rRNA* gene (1498bp). Lane M:100bp Marker, Lane 1-5: positive for the 16S rRNA gene; Lane 6: negative control.

***16S rRNA* gene sequencing**

Our study results were matched with the following accession numbers (MF079288.1, KC252600.1 and MT279533.1) and affirmed as *A. hydrophila* with 98-100% identity. The results revealed that 30(24.79%) of the samples were contaminated with *A. hydrophila*, the higher rate of isolation was registered in Sulaimania where (26.67%) of the samples showed positivity for *A. hydrophila* (Table 2).

Table 2 Isolation rates of *A. hydrophila* using different analytical methods.

City	Number of Samples	Positive by culturing methods		Positive by the Vitek 2 system		Positive by sequencing analysis	
		No.	%	No.	%	No.	%
Duhok	51	18	35.29	13	25.49	12	23.53
Erbil	40	14	35.00	12	30.00	10	25.00
Sulaimania	30	10	33.33	9	30.00	8	26.67
Total	121	42	34.71	34	28.10	30	24.79

Antibiotic susceptibility test

The results of the antibiotic susceptibility test for the (30) molecularly confirmed isolates showed that the highest resistance was recorded for cephalothin, amoxicillin and tetracycline at the rate of (90%), (83.33%) and (83.33%) respectively, and most of the isolates showed sensitivity to ceftriaxone, cefixime, nitrofurantoin, ciprofloxacin, chloramphenicol and trimethoprim at different percentages (Figure 4).

Discussion

In the present study, we investigated the prevalence of *A. hydrophila* in food (chicken meat) samples collected from the three cities located in northern of Iraq (Duhok, Erbil and Sulaimania). We also aimed to demonstrate their antimicrobials susceptibility profile.

The results of the study revealed that 24.79% of poultry meat samples were contaminated with *A. hydrophila*. The study result is in agreement with the results of Rather et al. (2014) who found that 25% of

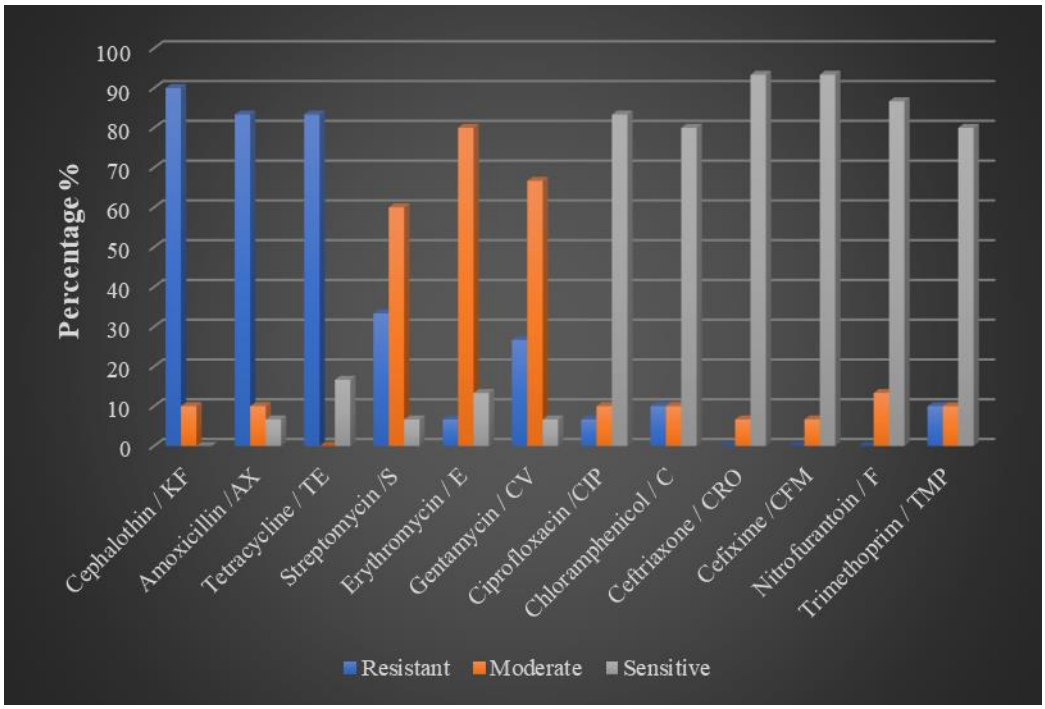

Fig 4. Antimicrobial susceptibility histogram for A. hydrophila isolated from chicken meat samples.

chicken meat samples were contaminated with *A. hydrophila*.

Comparing the results of our study with those from other cities in Iraq, it is unfortunate that there is not much research on the isolation of *Aeromonas hydrophila* from poultry meat in Iraq. However, there is one study conducted in Thi-Qar city, southern Iraq, which is the study carried out by Musa and Ahmed (2017) which identified the presence of *Aeromonas hydrophila* in 18.4% of samples.

Elmanama & Ferwana (2011) isolated these bacteria from 16.7% of chicken meat samples collected from Gaza Strip, Palestine.

Dallal et al. (2012) isolated *A. hydrophila* from 32.4% of fresh chicken meat samples and from 14% of frozen chicken samples collected from retail outlets of Tehran- Iran. The prevalence of *A. hydrophila* in frozen chicken confirms the fact that these bacteria can survive, grow at low temperatures and produce enzymes and exotoxin (Sheir et al. 2020).

The higher incidence was reported by Rajakumar et al. (2012), who found *A. hydrophila* in 66.6% of chicken meat samples; this result was attributed to the contamination of carcasses by washing water because in the same study they found that 40% of water samples were contaminated with *A. hydrophila*.

Antimicrobial resistance has become a significant challenge worldwide. The frequency of bacterial resistance to different antibiotics in food and food products has increased throughout the last few years, certain factors contribute to the rise in antibiotic resistance including; overuse and misuse of antibiotic in animal production (Yousif & Jwher 2019), using antibiotics as growth promotor (tetracycline) and therapeutically (cephalothin and amoxicillin) particularly in poultry production, so that consumers can potentially consume animal products that carry resistant bacteria (Endale et al. 2023).

Antibiotic susceptibility test results in this study showed that 90% of the isolates were resistant to cephalothin, and 83.33% were resistant to amoxicillin, and tetracycline, and most of the isolates showed sensitivity to ceftriaxone, cefixime, nitrofurantoin, ciprofloxacin, chloramphenicol and trimethoprim at different percentages. Resistance to amoxicillin and cephalothin (beta-lactam antibiotics) could be attributed to the lactamase enzyme produced by the expression of chromosomal lactamases (Hafez et al. 2018).

In agreement with our results, Dallal et al. (2012) found that *Aeromonas* spp. isolated from chicken samples were strong resistant to ampicillin, cephalothin and tetracycline at 90%, 80% and 70% respectively, in the same study they found approximately similar results regarding sensitivity to chloramphenicol and trimethoprim.

Higher rate of resistance to ampicillin was found by Elbehiry et al. (2019), who found that 95% of *A.*

hydrophila isolated from chicken samples were resistant to ampicillin.

In a study aimed to investigate the prevalence of different *Aeromonas* species in domestic birds. Mourad et al. (2022) stated that *A. hydrophila* isolates were more resistant to antibiotics than the other *Aeromonas* species. The existence of resistant isolates of *Aeromonas* is of great importance to public health since these bacteria are ubiquitous in soil and water systems and have the opportunity to interact with bacteria from a variety of different sources; therefore, *Aeromonas* species may be a reservoir for "resistance genes" and contribute toward its potential spread (Meng et al. 2020); or through its ability to produce biofilms. (Jwher et al., 2013; Yousif et al. 2023)

Our findings reflect the high levels of resistance of *A. hydrophila* to antimicrobials. It is believed that in the developing countries including our region, there is a better access to drugs and widespread uses of antibiotics either as growth promoters or as therapeutic agents to treat various diseases (Yousif & Jwher 2021).

This research not only highlights the health risks posed by the presence of bacteria in food of animal origin, but also emphasizes the need for enhanced monitoring and implementation of necessary hygienic standards in poultry management to mitigate the risk of foodborne illness.

Conclusions

The results of the current study indicated that chicken meat samples were contaminated with *A. hydrophiila*, which may be a source of transmission of these pathogens from food to humans. The current findings demonstrate that the use of PCR for amplification of *16sr RNA* gene and sequence analysis offers a rapid, sensitive and specific method to assess the presence of *A. hydrophiila* in food samples. Most of the isolates were resistant to many antimicrobial agents used in the study. It is completely necessary to give more attention to these bacteria in the field of food microbiology because they are able to produce toxins and grow under low temperatures and a broad spectrum of environments. Effective hygienic and sanitation procedures in the meat products industry are needed to minimize the health risks of pathogenic bacteria.

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Conflict of interest

The researchers confirm that there is no conflict of interest and no infringement of the property and intellectual rights of others.

Authors Contributions

The study was designed by all researchers who participated in the work, including sampling, investigation, analyzing the results and writing the manuscript, including the financial costs.

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