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Molecular identification of *Pasteurella spp*. in poultry serum and liver samples by PCR in the central region of Iraq (Babylon)

Asaad Mohammed Merzah, Abdolghaffar Ownagh*, Amir Tukmechi

Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran

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Abstract

Pasteurellosis is a zoonotic disease that affects a variety of animal species. It is caused by bacteria belonging to the *genus Pasteurella*, which can result in economic losses for the poultry and livestock industries. The most prevalent modes of transmission of the bacterium to humans are through bites or scratches inflicted by infected animals and contact with the mucosal membranes or blood of said animals. Humans can contract pasteurellosis by being bitten or scratched by infected animals, or through contact with their mucus or blood. A cross-sectional study was conducted in mid-2023. Two hundred sixty serum samples and 175 liver tissue samples were collected from ten broiler farms (age range: 30–42 days) in the central regions of Iraq for during the year 2023. The samples underwent DNA extraction using a DNA extraction kit from Pooya Gen Azma, Iran. Identification up to genus *Pasteurella spp*. was done using the *16S rRNA* gene. The primers were designed using Amplifix version 1.5.4 software. In this study, the PCR amplification was done on serum and liver DNA samples. The results indicated that 63 of the serum samples (i.e., 24.23%, [95% CI: 19.42 - 29.79]) and 26 of the liver samples (i.e., [14.80%, 95% CI: 10.35- 20.90]) were positive for the *genus Pasteurella*. The findings of this study help to elucidate the existing gaps in knowledge regarding *Pasteurella spp*. Such research will undoubtedly contribute to the greater overall protection of human health.

Introduction

Pasteurella spp. is a group of Gram-negative bacteria that commonly affect poultry, causing respiratory diseases, septicemia, and other infections. These pathogens pose a significant threat to the poultry industry, leading to economic losses and impacting public health due to their zoonotic potential (1). The emergence and spread of antibiotic-resistant *Pasteurella* spp. strains have further exacerbated the problem (2), necessitating an urgent need to understand their prevalence and antibiotic susceptibility patterns. The clinical manifestations of respiratory disease caused by *P. multocida* include cough, fever, dyspnea, and chest pain. While pneumonia represents the most prevalent form of infection resulting from *P.*

***Corresponding author:** *a.ownagh@urmia.ac.ir*
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multocida, other potential outcomes include tracheobronchitis, empyema, and lung abscesses. *Pasteurella* is a component of the mucous flora of the respiratory system, and the disease frequently manifests in response to stressors such as the accumulation of livestock, exposure to cold temperatures, transportation, or the presence of concurrent infections (3).

When initially introduced into the body, *Pasteurella* can exhibit pathogenic characteristics, and in such instances, transmission between animals is often facilitated by small droplets or contaminated water and food (4). Ticks and fleas are natural carriers of pasteurellosis. In contrast to mammals, this bacterium is not a natural component of the avian flora. Its presence in a bird is indicative of either an acute or chronic disease state. Hemorrhagic septicemia diseases in cattle, sheep, and fowl are caused by highly invasive species of bacteria, which act as the primary cause of disease in secondary infection. The diseases are characterized by their high level of invasiveness. It has been established through research that the majority of instances of respiratory disease in cattle and pigs are the result of bacterial invasion (5). The investigations also identified the specific outer membrane proteins (OMPs) present in *P. multicida* as playing an essential role in the onset of the disease (5). Infection of poultry with *P. multocida* can result in the development of fowl cholera, which represents a significant economic concern in commercial production. Infections caused by *P. multocida* can present in forms of acute, acute, and chronic infections. The histological features, clinical signs, and macroscopic lesions associated with these infections in poultry and pigs often lack distinctive characteristics. Which can lead to misdiagnosis these infections may be incorrectly identified as other respiratory conditions. Such as those involving inflammation of the upper respiratory tract, pneumonia air sac colitis, polyserositis, and septicemia. Consequently, accurately identifying the causative organism is crucial for making an appropriate diagnosis (6).

Pasteurella multocida is capable of causing disease in numerous animal and avian species. It is a primary agent responsible for respiratory infections in animals and represents an essential zoonotic disease (4). In a clinical context, if an elderly patient presenting with a chronic lung condition has a history of exposure to cats or dogs, the clinician must be cognizant of the potential for *Pasteurella* to be a causative agent of their pulmonary illness (7). A study conducted in Iraq on the prevalence of pasteurellosis showed that the percentage of *Pasteurella multcida* diagnosed bacteria from animals and humans was 29.4% and 16.9%, respectively (8). The central region of Iraq, particularly Babylon province, represents a significant agricultural area with a thriving poultry industry (9), by utilizing specific primers targeting conserved regions of the bacterial genome and known resistance genes (10), PCR allows for accurate and rapid detection of the target organisms and resistance markers. Additionally, PCR can be combined with sequencing techniques to further characterize the identified *Pasteurella spp*. strains (11). The objective of this study was to identify the presence of *Pasteurella spp.* in serum and liver samples from the Iraqi Babylon area, specifically targeting gene *16S rRNA*.

Materials and Methods

Study area

Iraq's geographical coordinates are between 29º and 38 ° north in latitude and 39° and 49° east in longitude, with a small portion extending westward into the latter zone (see map). Except for the northern regions, the climate of the country is predominantly arid, characterized by cool to cold winter temperatures and dry, warm, and sunny summers. During the summer months, temperatures in most regions of the country reach up to 40 °C, with some areas experiencing temperatures as high as 48 °C. The temperature rarely surpasses 21 degrees Celsius during the winter season, with the most frigid conditions reaching between 15 and

19°C. Nighttime temperatures typically range between 2 and 5 °C.

Fig. 1. Study area.

Sampling

This was a cross-sectional descriptive study carried out in 2023. A total of 260 serum samples and 175 liver tissue samples of chickens with clinical signs suspected to be pasteurellosis were collected from 10 selected broiler farms located in the central region of Iraq, Babylon. Samples were transported to the microbiology laboratory of the Faculty of Veterinary Medicine on ice after collection. In the laboratory, samples were cultured in a TSB broth medium and incubated for 24 hours before DNA extraction analysis.

DNA extraction from samples

DNA was extracted from all the samples using a commercial DNA extraction kit (Pooya Gen Azma, Iran). One hundred microliters of serum and 200 mg of liver tissue were added to a 1.5-ml microtube. Two hundred microliters of MR buffer were added to it, and the sample was gently mixed. The tubes were then left at 0°C for 2–3 min. After incubation, the samples were centrifuged at 9000 rpm for 4–5 min, and the supernatant was decanted. The sediment obtained was used in the following extraction steps. For the subsequent step, the pellet was re-suspended by adding 200 µl of MR buffer to it, followed by another centrifugation at 9000 rpm for 4-5 minutes. The suspended sediment was added with 200 µl of MI buffer. After that, 50 µl of MII buffer was added to the resuspended sediment, the mixture inverted gently twice and then incubated at room temperature for 20-30 minutes until the solution became completely clear.100 µl of MIII buffer was added to the pellet, after which the tubes were gently inverted about 10 times until the liquid in the pellet turned white. The microtubes were then centrifuged at 13,000 rpm for 10 minutes, after which the supernatant was carefully aspirated and transferred into a newly labeled microfuge tube. To the DNA solution obtained, add an equal volume (100 µl) of 96% ethanol, mix, and then centrifuge in a microcentrifuge at 13,000+ rpm for 5 minutes to precipitate the DNA. Then, another 200–500 µl of ethanol was added, and after that, the microtubes were centrifuged once more at 13,000 rpm for 2–3 minutes. The ethanol was poured out and the

sediment was dried, with the time depending on the room temperature. Usually, dry it for 2–3 min. Finally, add 20–50 µl of solubilizing buffer to the sediment to fully dissolve the DNA, and store it at −20°C until PCR analysis. For validation of the extraction method and concentration assessment, absorbance at 260 nm was measured using a Nanodrop spectrophotometer for 10 randomly selected samples, and the value obtained was recorded. The DNA/protein purity was calculated.

PCR for molecular detection of Pasteurella spp.

Identification at the genus level was done by blasting the sequences with other members of the *Pasteurella* spp. genus. The primers were designed with Amplifix version 1.5.4. Software. The primers and temperature programs are detailed in Table 1.

Table 1. The primer list and temperature programs for amplifying the *16S rRNA* gene of the *Pasteurella spp.*

Target	Name	5'----3'	Condition	size (bp)
16S rRNA	*Pasteurella spp.*-F	TAGCGGTGAAATGCGTAGAG	95c for 30m, 95 c for 30s, 58 c for 30s, 72c for 45s and 72c for 7 m. (38)	353
	Pasteurella spp.-R	TAAGTTCCCGAAGGCACAAG		

PCR amplification of 16S rRNA gene

The polymerase chain reaction (PCR) was conducted in a 25-µl reaction volume, comparising 4 µl of template DNA, 1 µl of each primer (initial concentration 20 µM), and 12.5 µl of the master mixture (Red Master mix 2X Taq,1.5mM $MgCl_2$, Ampliqon, Danmark), with sterile distilled water used to achieve the total volume. A thermocycler (Quanta Biotech, UK) was employed to define the thermal cycling and PCR program following Table 2. The PCR products were subsequently electrophoresed on a 2% agarose gel comprising a safe staining solution (Labnet, ENDURO, USA), and visualized with the aid of a Genius Gel Documentation apparatus (Syngene Bio-Imaging, UK) (Figure 2).

Sequencing

Positive samples underwent sequencing using the Sanger method to determine the nucleotide bases at Pishgam Biotechnology Company in Tehran. These sequences were then analyzed and subjected to BLAST comparison on the NCBI website. A combined sequence of 353 nucleotides of the *16S rRNA* gene was used for the construction of a phylogenetic tree. Mixture degenerate primers are blends of related primer sequences that accept several variations at specific positions under the genetic code's framework.

Phylogenetic analysis

A phylogenetic tree was built using a combined sequence of 353 nucleotides (Figure 3). The evolutionary analysis revealed that strains OR934739 and OR939278 were the subjects of this study. The isolate is mentioned, and its corresponding marker describes the method by which it was obtained from the current investigation. The tree is derived from the sequence of the *16S rRNA* gene. The evolutionary history was documented using the Neighbor-Joining method, as described in reference (12). The optimal evolutionary tree is described here, depicted at a proportional scale, with branch lengths expressed in the same units as those employed to infer the evolutionary tree. The evolutionary distances were determined using the Tamura 3-parameter method, which is 12 units of base substitutions per site. The percentage of sites with at least one unambiguous base in at least one sequence for each of the descendent clades is displayed adjacent to each internal branch in the tree. This analysis included 25 different nucleotide sequences. All of the ambiguous positions were erased for each pair of sequences (the option of deleting multiple sequences). The final dataset had a total of 368 positions. Evolutionary studies were conducted in MEGA11 (4).

Results

This study was conducted in Iraq in 2023, analyzing 260 serum samples and 175 liver samples from broilers obtained from the central region of Iraq (Babylon) by the PCR method. In the serum samples, 63 out of 260 samples were positive for the *Pasteurella* genus (i.e., 24.23%, 95% CI: 19.42 - 29.79), while in liver samples, 26 out of 175 tested positive (i.e., 14.80%, 95% CI: 10.35- 20.90) (Figure2). Then the positive samples were sent to Pishgam Company in Tehran, Iran for sequencing, and after BLAST analysis on NCBI, the samples showed the highest similarity to *Pasteurella moltocida*, which was registered in GenBank with accession numbers OR934739 and OR939278). A phylogenetic tree was then drawn for them (Figure 3).

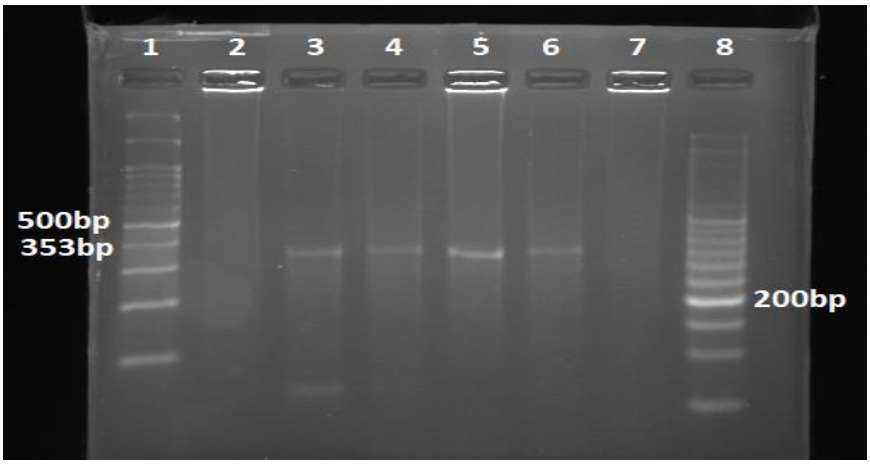

Fig. 2. Agarose gel image of the amplified fragment of *Pasteurella* spp. The 16srRNA gene (353 nucleotides) was amplified by PCR. The first lane is a 100-bp ladder, and the eighth lane is 50-bp ladder (both from Smobio Technology Inc., Taiwan). Lanes 3 to 6 are positive for *Pasteurella* spp., lane 2 is a negative control, and lane 7 is a negative examples.

Discussion

As a majority of re-emerging and emerging infectious diseases have a zoonotic pattern, the probability of humans interacting with both domestic animals and wild species is increasing. As a result, the risk of humans getting sick from animals is increasing. The Pasteurellaceae family is composed of bacteria that are common in animal populations. They are typically found as part of the normal microbiota, or a collection of bacteria that live and thrive in a particular region, the oral, nasopharyngeal, and upper respiratory tract. Significant portions of the *Pasteurella* genus are pathogens that are opportunistic and cause endemic disease as well as increasingly associated with epizootic outbreaks. The most common route of transmission from animals to humans is through animal bites or contact with nasal secretions. Of all the isolates of *Pasteurella* that have been observed in human infections, *P. multocida* is the most prevalent (13). Species of *Pasteurella* are involved in the causation of a variety of endemic and epizootic diseases that have a significant economic burden on a variety of domestic and wild animals and birds. *P. multocida* is a common parasite or predator that is found in the upper respiratory passages of most animals, including livestock, humans, and nature. *P. multocida* isolated from birds include chickens (14-19), as well as turkeys (20, 21), other wild birds (22, 23), cattle and bison (22, 24), and swine (22, 25). Additionally, the bacterium has been identified in a range of other animals, including rabbits (26, 27), dogs (22, 28), and cats (domestic house cats as well as large wild

cats, such as tigers, leopards, cougars, and lions) (22). It has also been isolated from goats (22), chimpanzees (29), marine mammals (seals, sea lions, and walruses) (30), and even Komodo dragons (31, 32). The presentation and associated pathological symptoms of *Pasteurella* infection, or "pasteurellosis", are variable and range from symptomatic or mild chronic inflammation of the upper respiratory passages to acute, often fatal, pulmonary disease and disseminated infection.

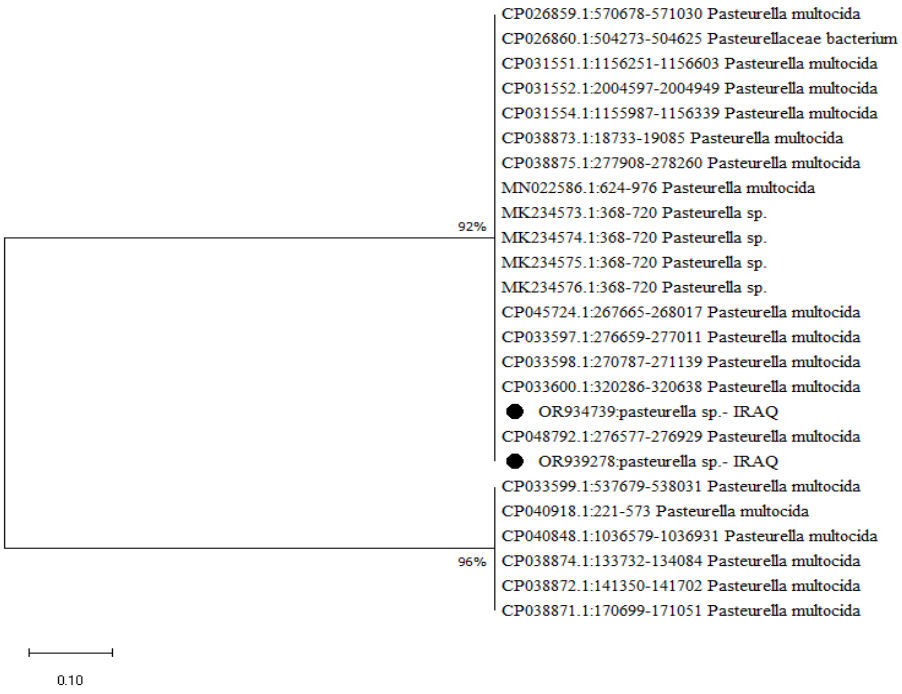

Fig 3. The evolutionary history of the 16SrRNA gene's sequences from *Pasteurella spp*. The present study's findings and those in GenBank that are derived from different numbers of accessions; the numbers of accessions are after the isolate's names. The 16SrRNA gene sequences that were obtained in this study are noted by bold triangles. The tree was constructed using the Neighbor-Join method in MEGA 11; the values at each branch point are bootstrap. The numbers above the branch are the results of 1000 replicate experiments. All of the sites that had insertions or deletions were removed from the analysis.

Table 2. The research results were subjected to statistical analysis based on serum or liver samples ($p<0.05$)).

Variable	Frequency	PCR-Positive (%)
Serum sample	260	(63)24.23%
Liver sample	175	(26)14.85%
Total	435	(89)20.45%

There is a deficiency of studies about the infection of pasteurellosis in Iraq. The bacteriological and serological methods that have been employed primarily identify *P. multocida*. However, several reports have documented the presence of *P. multocida* in field animals using these methods alone. A study on pasteurellosis in the respiratory system was documented in Iraq during the winter season; this infection was present with a prevalence of 14% in the infected group and 4% in the healthy group (33). Other investigations have reported that *P. haemolytica* was isolated from sheep in Baghdad at a frequency of 8.57%, (34). In addition to isolating *M. haemolytica* during an epidemic in mountain goats, deer, and gazelles, the pathogen was found in 68.0% of nasopharyngeal samples and 3.17% of blood samples (33).

A review of the literature reveals that studies of the pathognomonic features of *P. multocida* isolate among various avian species have demonstrated considerable genetic variations among *P. multocida* strains from each avian species (35). The aforementioned alterations can be ascribed to several factors, including variations in the host's immune system, environmental conditions, and modes of transmission. These cases have a significant impact on the pathogenesis, diagnosis, treatment, and prevention of *P. multocida* infections in diverse avian species (36).

In the study conducted by El-Demerdash., et al. 2023 (36), A total prevalence of 9.4% (30/317) was observed for *P. multocida* among the sampled birds. The majority of the isolates were determined to be of capsular type A (96.6%, 29/30), a result that aligns with the findings of previous studies in this field (37, 38), It discovered capsular type A to be the most common among avian strains and isolated *P. multocida* in comparable percentages. Notably, 3.4% (1/30) of the isolated *P. multocida* from quails had capsular type D, a serogroup that is thought to be uncommon (39, 40). This discovery demonstrates the variable prevalence of the serogroups in instances of respiratory disease, which appears to be dependent on the region (41). Climate change is significant in the environmental sphere and has the potential to have an effect on the transmission of this exceptional serotype and the propagation of this infectious disease (42), *Pasteurella multocida* is more likely to have a successful and extensive presence in warm, damp environments. As a result, climate change is expected to lead to a higher degree of isolate prevalence among different avian species (43).

In this study, a phylogenetic analysis was conducted utilizing *16S rRNA* fragments of species isolated from serum and liver samples. Phylogenetic analysis of *Pasteurella* strains from different serum and liver samples showed their evolution closely with those found in other animal species of animals and, therefore, closely related to strains associated with humans. This close relationship is likely due to interaction between infected animals and humans raising the risk of zoonotic infections, such as Pasteurellosis, among humans. There is a complete lack of *Pasteurella* surveillance in the region, which indicates very strongly the potential health risk this bacterium and its diseases carry. The result further revealed a strong phylogenetic linkage with strains that were previously narrated in Iraq, China, India, and Turkey which was in line with the previous studies.

Conclusion

The findings of this study help to elucidate the existing gaps in knowledge regarding *Pasteurella* spp. in poultry samples from the Babylon region in Iraq. The findings of this research will contribute to our understanding of the epidemiology of *Pasteurella* spp. infections and

guide the effective control of diseases and the management of antibiotics on poultry farms. Additionally, the data on the prevalence of *Pasteurella* spp. in poultry serum and liver samples can inform the development of effective disease management strategies. Awareness of the prevalence of *Pasteurella* spp. in the central region of Iraq (Babylon) can assist poultry farmers and veterinarians in the implementation of preventative measures, early detection methods, and targeted interventions to control the spread of the bacteria and minimize disease outbreaks. The research findings from this study have the potential to be applied in of several different ways. *Pasteurella* spp. has the potential to transmit diseases to humans, as they are zoonotic. A deeper comprehension of the prevalence and antibiotic resistance profiles of *Pasteurella spp*. strains in poultry may facilitate the mitigation of public health concerns.

Ethical approval

This work was conducted in accordance with the guidelines and standards of the Animal Research Ethics Committee of Urmia University. Serum samples were collected from live animals aseptically, while liver samples were collected from the necropsies of deceased animals. Thus, there was no need to obtain an ethics code for this study.

Competing of Interests

The authors declare no conflicts of interest.

Acknowledgments

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