Comparative polypeptide mapping of whole cell extract from vaccine and field avian strains of *Pasteurella multocida* using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

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ABSTRACT

Introduction Fowl cholera is a significant global disease affecting birds, caused by *Pasteurella multocida*. Vaccination is the primary prevention strategy. This study aimed to investigate potential molecular-level inter-strain variations between the avian vaccine strain and wild isolates of P. multocida.

Methods Three *P. multocida* field isolates collected from deceased birds, along with the vaccine strain Razi Pm0002 Asara were assessed. Protein analysis of the whole-cell extract from bacterial mass cultures was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Results The SDS-PAGE profiles revealed a variety of major and minor polypeptide bands, with molecular weights ranging from 36 kDa to 105 kDa. A high level of common dominant proteins was detected across the strains. Notably, only five major and minor protein bands exhibited differences between the vaccine strain and field isolates. The identified polypeptide variations indicate a clear opportunity for further comprehensive proteomic and genomic analyses to improve the currently available fowl cholera vaccine in Iran. Furthermore, all three field isolates displayed identical protein profiles, a likely (but not certain) confirming of their classification as the same serotype. Additionally, we assessed the effects of three different liquid broth culture media (PPLO, BHI, and TP) on the mass culture of the vaccine strain and found no changes in the protein profile of the resulting bacterin.

Conclusion From the perspective of the vaccine manufacturer, these preliminary findings suggest that any of the three examined media may be used interchangeably for large-scale bacterin production, though further evaluations are warranted to validate this conclusion.

Keywords: Fowl Cholera, Iran, Protein, Serotype, Vaccination

1 Introduction

Pasteurella multocida, a gram-negative aerobic bacterium characterized by its short rod/coccoid shape, is responsible for causing hemorrhagic septicemia (HS) in ruminants, fowl cholera (FC) in avian species, and atrophic rhinitis in swine (1). Between 2005 and 2019, approximately 106 million cases of HS were reported among ruminants across 41 countries. Of these, roughly 100,000 animals (0.1% of those affected) succumbed to the disease, resulting in an annual economic loss estimated at USD 700,000 (2). Fowl cholera affects a wide range of avian species globally, presenting symptoms that can vary from chronic or localized infections to acute septicemia, which can lead to morbidity and mortality rates as high as 100%. The primary strategy for disease prevention is vaccination with inactivated or live vaccines; however, these biological options have

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limitations that can render them less than ideal. The poultry sector continues to expand and industrialize on a global scale (3). In Iran, poultry production is a vital component of national food security, with the trade—almost entirely managed by the private sector—contributing significantly to the country's GDP (projected at 2.5% in 2025). Approximately 1.2 billion chickens are raised, generating an annual revenue of around 9 billion USD. Most, if not all, of the northern provinces of Iran, such as Gilan, Mazandaran, and Golestan, are reported to be endemic to the disease (4-6). In 1972, Bozorgmehri Fard and colleagues were among the first to highlight the seriousness of this disease in these regions (7). Some researchers believe that *P. multocida* is the primary culprit behind the mysterious illness outbreaks that sporadically impact northern Iran. The disease severely affected a substantial number of migratory wildlife birds in the Miankaleh nature conservation area recently in 2020 (8) and 2021, although it had been documented there as early as 2006 (4).

The observation that some vaccinated animals remain unprotected against subsequent infections with *P. multocida*, even shortly after vaccination, may indicate either insufficient immunity conferred by the bacterin vaccine or the emergence of infections caused by strains that differ antigenically from those used in the vaccine formulation (9). Consequently, the development of an effective vaccine for any specific area relies on selecting vaccine production strain(s) that are both antigenically similar and immunologically homologous to the strains that are epidemiologically prevalent in that region (10).

Traditionally, the diversity of *P. multocida* has been assessed through serotyping, which is based on the antigenic properties of the capsule polysaccharide and the composition of lipopolysaccharides (LPS) (1). However, this method is often time-consuming and labor-intensive, as it necessitates specific antisera that can be challenging to prepare.

To address these challenges, alternative methods such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which analyzes bacterial protein profiles, and random amplified polymorphic DNA (RAPD) have been employed for biodiversity analysis (1) across various bacterial pathogens. Despite the studies conducted on *P. multocida*, the full extent and richness of its biodiversity have yet to be comprehensively analyzed (7, 11, 12).

Polyacrylamide gel electrophoresis (PAGE) is a widely utilized technique across numerous scientific disciplines, including biochemistry, molecular biology, forensics, biotechnology, and

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genetics. This method facilitates the separation of biological macromolecules, particularly proteins and nucleic acids. The separation occurs according to electrophoretic mobility (13), which is influenced by the length, conformation, and charge of the molecule (13). Over the past several decades, SDS-PAGE has gained prominence in bacterial classification (14) and the identification of protective antigens (15). This technique is relatively straightforward to perform and can accommodate a large number of samples in a single run (16).

The application of SDS-PAGE for polypeptide mapping of *P. multocida* isolates has revealed varied protein band profiles, with sizes ranging from 16 to 104.71 kDa (17, 18). Among these, the immunoblot assay identified several immunogenic bands, specifically at 82 kDa, 44.37 kDa, 37 kDa, 32 kDa, and 30 kDa (19).

In this study, we aimed to investigate potential inter-strain variations at the molecular level between the avian vaccine strain and field isolates of *P. multocida*, focusing on protein fractions using the SDS-PAGE technique.

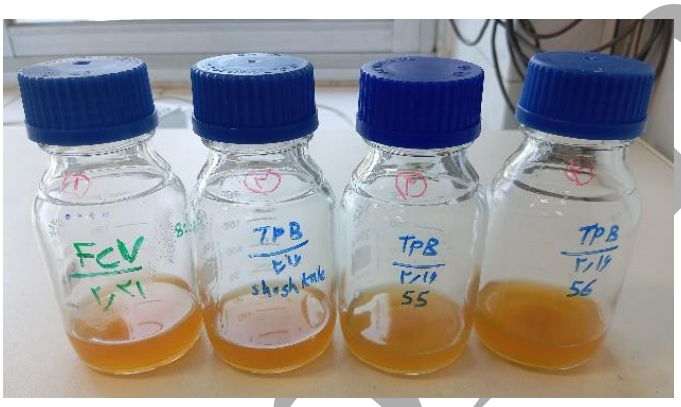
2 Materials and Methods

2-1 Isolation and Identification Four indigenous *P. multocida* strains were utilized, including two recently collected (2024) isolates from birds that succumbed to fowl cholera (designated as strains 55 and 56) and the Razi Pm 0003 Shoushkaleh isolate, which was collected from a deceased bird circa 2015, along with the commercial vaccine strain Razi Pm 0002 Asara. The identity of these strains was confirmed through Gram staining, conventional biochemical tests, and KMTC-PCR (20).

2-2 Sample Preparation for SDS-PAGE Technique The Razi Pm 0002 Asara strain was obtained from a blood agar plate sourced from the Pasteurellosis Vaccine Production Department at RVSRI. Similarly, the other three strains were obtained on blood agar plates from the Avian Disease Diagnosis Department at RVSRI. These plates were then used to inoculate culture bottles. The bacterial biomass for all strains was cultivated by inoculating 50 mL of pure tryptose phosphate broth (TPB) in blue screw-capped 100 mL glass bottles (Fig 1).

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Fig 1. Mass bacterial culture of Razi Pm0002 Asara strain and Razi Pm0003 Shoushkaleh, 55, 56 *Pasteurella multocida* isolates in Tryptose Phosphate Broth medium.



Overnight incubation of the cultured bottles at 37°C continued until a culture optical density of 1.0 was reached. This strategy ensured the incubated culture bottles carried not less than 1.0 $\times 10^9$ CFU/ml cell mass of the bacterium. The bacterial lawn from each bottle was checked for purity by microscopy and culture on a blood-agar plate. To stop the growth and lyse the bacterial cells culture, bottles were subsequently submerged in a water bath and received a single round of heat-treatment at 65°C for 30 min. The complete inactivation of bacteria was tested by inoculation of blood-agar plates with 1ml of culture bottle contents and incubation at 37°C overnight.

For gel electrophoresis, the Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique was performed using a discontinuous buffer system in vertical mode. Briefly, whole cell proteins were analyzed with a 4% stacking gel and a 12% separating (resolving) gel. The resolving gel was prepared at a pH of 8.8, while the stacking gel was set to a pH of 6.8, and the tank/running buffer achieved a pH of 8.3 through the addition of NaOH and/or HCl solutions. Samples were mixed with a 2x treatment buffer (0.125 M Tris-Cl pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol) and heated for three minutes. Following a 5-minute centrifugation at 150 g, the supernatant was collected for loading. For each sample, 10 µL of the

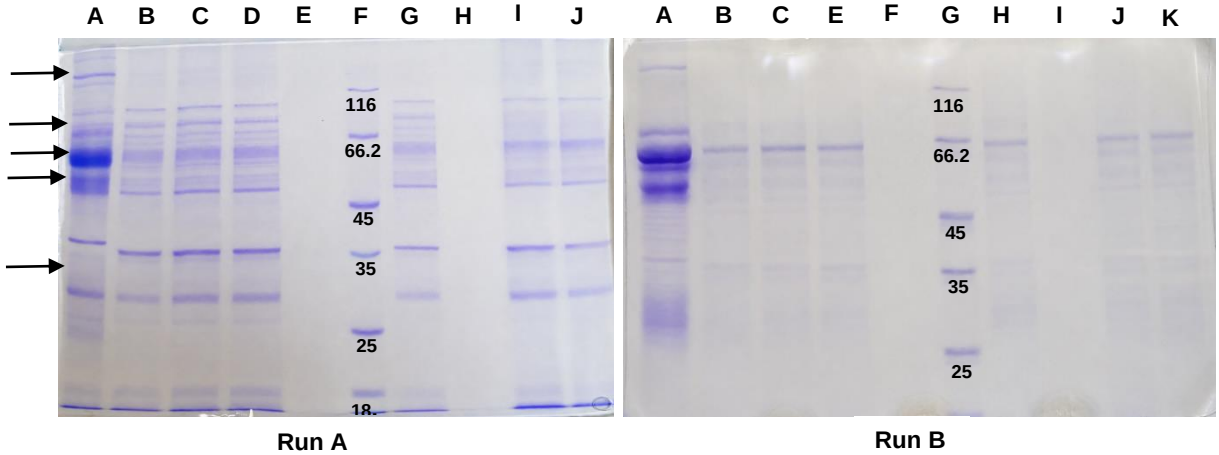
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prepared sample was loaded into the gel. Additionally, a molecular weight marker, ranging from 14.4 KDa to 116 KDa (Bio-Basic, BSM0431), was loaded into a separate well. The electrophoresis was conducted by applying 100 V for 60 minutes. After electrophoresis, the separating gel was immersed in a staining solution of Coomassie Brilliant Blue R-250 (Bio-Rad, 1610786), followed by destaining with Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad, 1610438). The gel was shaken continuously for several hours until clear protein bands were visible; it was then rinsed with distilled water and photographed in white light using a gel documentation system. Protein band molecular weights were estimated by visual comparison with the protein marker used. The entire SDS-PAGE experiment was conducted in quadruplicate to ensure accuracy of the findings and to achieve optimal resolution.

3 Results

An analysis of the protein profiles of field isolates and the vaccine strain of *P. multocida*, based on Coomassie blue-stained gels, revealed that the differences in polypeptide profiles among these isolates were minimal. The vaccine strain exhibited a total of 32 polypeptides with molecular weights ranging from 22 to about 140 kDa. The three field isolates, namely Razi Pm 0003, Shoushkaleh, and isolates 55 and 56, displayed identical protein profiles, also comprising 32 polypeptides within the same molecular weight range of 22 to about 140 kDa.

While most protein bands were detected in the upper segment of the gel between 45 and 116 kDa, notable differences in protein profiles between the vaccine strain and field isolates were identified. The most significant distinctions were observed at five positions corresponding to major proteins within the 45 to 95 kDa range. Nevertheless, three proteins, with molecular weights at the ranges of 90-95 kDa, 30-40 kDa, and 20-23 kDa, were common to all field isolates as well as the vaccine strain of *P. multocida* (see Fig 2).



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Fig 2 (Run A & Run B): SDS-PAGE Whole-cell protein fingerprinting of representative P. multocida isolates. Lane F contain the Protein Marker (Bio-Basic, BSM0431). Lane A: Razi Pm0002 Asara strain, Lanes B, H, I J and K: Razi Pm0003 Shoushkaleh, Lane C: isolate 55, Lane D: isolate 56. Protein marker band sizes (KDa) are marked.

4 Discussion

The protein repertoire profiling of *P. multocida* bacteria was conducted using SDS-PAGE, with measurements taken against a known protein marker. A review of the published literature on *P. multocida* reveals a varied number of polypeptide bands and a wide range of molecular weights reported from SDS-PAGE analysis of whole-cell bacterial mass. Numan (17) and Munir (21) identified 7, 6, and 8 bands within a molecular weight spectrum of 15 to 104.7 kDa. Similarly, Srivastava (22) and Sridevi (9) detected 12 protein polypeptides within the ranges of 16-150 kDa and 26 to 120 kDa in their whole-cell lysate analyses, respectively. Johnson (23) and Singh (24) reported 13, 12, and 15 bands in their investigations, respectively. In contrast, Lee (25) identified twenty-six polypeptides in the 14 to 200 kDa range, while Nawaz (26) discovered 31 bands ranging from 11 to 125 kDa.

In Iranian studies, Jabbari, utilizing a Coomassie blue-stained gel, reported over 30 protein bands ranging from 24 to 174 kDa in the whole-cell protein lysate of *P. multocida*. As illustrated in Fig. 2, and consistent with Jabbari's findings, we have similarly detected more than 30 bands in the whole-cell protein repertoire of both vaccine-derived and field isolates of *P. multocida*. As noted by researchers in this field, the findings of SDS-PAGE polypeptide separation are expected to exhibit considerable variability based on experimental conditions, techniques employed, and the background of the isolates (10). Additionally, researchers such as Irfanullah (27) argue that the culture medium used for bacterial mass growth and the sample processing strategies are significant factors contributing to the variations observed in the polypeptides detected by SDS-PAGE. In contrast, our findings indicate that the culture medium has little to no significant impact on the

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number or size of detectable bands. We observed no differences in the polypeptide bands produced by the Razi Pm 0003 Shoushkaleh strain when grown in Tryptose Phosphate (TP) broth, pleuropneumonia-like organisms (PPLO) broth, and Brain Heart Infusion (BHI) broth (Fig 2). However, we believe this observation merits further comprehensive investigation.

Utilizing SDS-PAGE to explore inter-strain differences in whole-cell preparations of the vaccine strain and various field isolates revealed two complex yet distinct protein profiles. While most of the major and minor proteins were shared between the profiles, several unique bands enabled differentiation between the two patterns. Notably, these differences included three major bands at approximately 50, 38, and 22 kDa, in addition to several minor bands primarily located within the 55–95 kDa range.

Regarding the molecular evaluation of the prepared inactivated bacterin, the SDS-PAGE results demonstrated that all three field isolates exhibited a distinct 38-kDa band with high staining intensity and an overexpressed pattern. The pronounced density of this band may indicate alterations in the protein composition or structure of these isolates, a finding that has been previously noted in other studies (28). To determine whether this dominant protein plays a significant role in antigenic cross-protection among *P. multocida* serotypes, further complementary studies at both molecular and immunological levels are necessary to validate this hypothesis.

A further intriguing observation from this study was the identification of an identical protein profile among isolates collected from the Miankaleh Peninsula over a ten-year period. The first isolate, referred to as Razi Pm 0003 Shoushkaleh, was received in our laboratory in 2014 after being collected from a deceased bird. The subsequent two isolates, designated 55 and 56, arrived in 2026, having been collected from two autopsied dead birds presumably infected by *P. multocida*.

5 Conclusion

If confirmed through molecular genotyping, the presence of a particular serotype of avian *P. multocida* that has been circulating in the region for over a decade may indicate the existence of an epidemiologically significant strain. This strain appears to have successfully dominated the area, showing resistance to vaccination efforts and continuing to affect avian hosts in the region.

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CRediT authorship contribution statement

ShKh developed the study concept and performed the laboratory work, including bacterial cultures, antimicrobial susceptibility testing and molecular analysis. **AZM** and **MSh** supervised, designed, drafted, and revised the manuscript. **PKh** and **KT** helped with data curation, writing, reviewing, editing and submitting of the manuscript for publication.

Data availability

Not applicable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics approval

Not required. No work with live vertebrates was conducted. Isolates used here were recovered from previously collected bacterial archives.

Declaration of Generative AI and AI-assisted technologies in the writing process

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