Serological Investigation of H5 Avian Influenza in Dairy Cattle: A Serological Survey and Review of Global Spillover Cases

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Abstract:

Avian influenza viruses, especially highly pathogenic H5 (HP), are significant pathogens that pose major risks to both animal and human health, as well as causing substantial economic losses in the poultry industry. Recent global reports have highlighted the spillover of highly pathogenic H5 avian influenza (AIV) into dairy cattle, raising concerns about the virus's zoonotic potential and transmission pathways. This study evaluated current knowledge of H5 AIV in cattle in Iran, critically analyzing serological data and assessing the virus's ability to cross species barriers through a survey of dairy farms in Qazvin and Isfahan provinces, in Iran, during 2024-2025. It involved testing 500 dairy cattle of various ages, breeds, and lactation stages. All samples tested negative for H5 antibodies using the hemagglutination inhibition (HI) test, which contrasts with international reports of H5 spillover in cattle, particularly in North America. On the other side the report notes the limitations of the HI test, including its low sensitivity to small levels of antibody in cattle sera, and recommends the need for supplementary diagnostic methods along with continuous surveillance for better detection, so the lack of detected antibodies may indicate no exposure or little viral circulation below detection levels. These results highlight the importance of ongoing surveillance, especially in regions close to wild bird flyways and intensive poultry farming areas, assessing risks related to the dairy farm workers during HPAI outbreaks. In addition these findings emphasize the need for targeted monitoring and risk assessment systems to manage emerging zoonotic threats, considering H5 AIV's capacity for cross-species adaptation and the rising frequency of global spillover events.

Keywords: Avian Influenza, Dairy cattle, Highly Pathogenic H5, Iran, serology,

Introduction:

Avian influenza viruses, particularly those in the H 5 subtype, have long been recognized as significant pathogens that can affect animal and human health [1]. The virus is traditionally associated with poultry and wild birds, and there has been an increasing capacity to infect various species on many occasions [2]. An outbreak of highly pathogenic avian influenza (HPAI) A (H 5) clade 2. 3. 4. 4. b, genotype B 3. 13, was first detected in dairy cattle in March 2024, with human cases among farm workers emerging in April [3]. The emergence of avian influenza H5N1 in dairy cattle has raised significant global concerns, particularly regarding its potential impact on both animal and human health. This was a new and concerning development, with recent serological studies suggesting that H5 avian influenza virus antibodies had been detected in cattle, thus raising a serious possibility of exposure or infection [4]. It is believed that contaminated milking tools and movements of dairy cows are major routes of virus spread within a farm. This new host system raises fundamental epidemiological questions regarding the transmission dynamics of H5 avian influenza and its zoonotic potential [5]. Currently, this virus has infected dairy cattle herds across 17 states in the United States [6]. While typically a self-limiting infection that resolves in 1–3 weeks, a large proportion of lactating cows in an affected herd (3–20%) can become sick, leading to significant drops in milk yield (20–100%) in individual animals and resulting in considerable impact on overall herd health and productivity. Additionally, serological investigations on one US dairy farm indicated a large proportion (83.7%) of subclinical infections, which may complicate detection and disease control(1). Mortality is generally low (less than 2%), although the case fatality rate may approach 7% among clinically affected lactating animals. The presence of this disease in cattle has raised many concerns regarding research on existing serological methods and warrants further investigation [7]. The serological studies show a snapshot of previous virus exposures, shedding light on prevalence, geographic distribution, and risk factors linked to H5 infections within cattle populations [8]. Not all subtypes of avian influenza effectively infect mammals; however, HPAIV H5 and H7 viruses occasionally break species barriers and infect humans, with high fatality. Significantly, the 2. 3. 4. 2.3.4.4b viruses have been reported in mortality or outbreaks in marine mammals or other wild mammals, indicating that several mammalian species, including seals, foxes, and bears, are vulnerable to the H5 clade 2. 3. 4. 2.3.4.4b and probably acquired the virus through direct or indirect contact with infected birds[9]. To date, several serological studies have been conducted to detect this virus in dairy farm workers and dairy cattle [10]. This type of monitoring is vital for livestock and is also critical as a public health consideration for changes within the host range of avian influenza viruses.

This study aimed to investigate the serology and determine the presence of antibodies against the highly pathogenic avian influenza (H5N1) virus in two major dairy cattle-producing regions of Iran,Isfahan and Ghazvin provinces, to assess the regional status and decision-making for preventing the outbreak of a potential pandemic.

Material and methods:

A serological study was conducted from 2024 to 2025 to assess the presence of the H5 avian influenza virus (AIV) antibodies among dairy cattle herds in Qazvin and Isfahan provinces, Iran. It included 500 serum samples from large and small herds and backyard cattle comprising different age, gender, and breed groups and production categories. The Samples collected from some lactating Holstein cows were categorized into the high production group. The majority of the cows had calved three or more times, and some samples included cows in early pregnancy (less than three months). Regarding vaccination, there was no history of vaccination against AIV; they were all immunized against Foot-and-Mouth Disease (FMD) and Brucellosis. The selection of the herds was carried out in such a manner as to ensure that the chosen ones represented the whole population based on their geographical distribution and management styles.

Blood samples from the animals were extracted from the jugular vein into sterile vacutainer tubes and were then immediately set for transport to the laboratory under cold-chain conditions. After the blood samples had been left to clot in the laboratory at room temperature, the sera were separated by centrifugation for 10 minutes at 3,000 rpm. The obtained sera were kept at -20°C until HI analysis.

HI test was performed according to the WOAH (formerly OIE) standard protocol for detecting antibodies against H5 AIV. Sampled sera were incubated at 56°C for 30 minutes to remove nonspecific Inhibitors, then each serum was twofold serially diluted with PBS (pH 7.2).

The test was performed with 4 HAU of H5 AIV antigen(H5N1,GD Animal Health) and 0.5% chicken RBCs in a 96-well U-bottom microtiter plate. After adding the H5 AIV antigen, the plates were kept at room temperature for 30 minutes before adding chicken RBCs, and kept again for 30 more minutes at 4°C. The highest serum dilution showing no hemagglutination was recorded as HI titer. Titers ≥ 16 ($\log2 \geq 4$) were considered positive for H5 AIV antibodies.(2, 3)

Data Analysis:

The data were imported into Microsoft Excel for statistical analysis by Using SPSS version 26, Descriptive statistics were calculated to summarize the H5 antibodies prevalence in the study population, which adopted stratification by province, herd size, and age group. Chi-square or Fisher's exact tests were performed to evaluate associations between seropositivity and categorical variables such as herd size or age group. P-values below 0.05 were taken as significant.

Results:

The HI test results revealed that none of the 500 serum samples were positive for H5 AIV antibodies. The titer in all sera was 0, and none of the samples had a titer above the cut point of 16, which implies no detectable level of antibodies against the H5 AIV among the studied population. Descriptive statistics were employed to present summaries of the prevalence of H5 antibodies stratified by province, herd size, and age group. Chi-square and Fisher's exact tests were

applied to compare the relationship of seropositivity with categorical variables such as herd size and age group. There was no seropositivity, and these statistical tests yielded no significant relationships.

Discussion:

The zoonotic potential of H5N1 has been a major concern, with occasional infections reported in both dairy workers and dairy cattle (4). Serological studies have played a key role in detecting and tracking (monitoring) the spread of H5N1 among dairy cattle. The current serological study, conducted between 2024 and 2025 in Qazvin and Isfahan provinces of Iran, represents the first systematic assessment of H5 avian influenza virus (AIV) antibodies in dairy cattle in this region. All 500 serum samples tested negative for H5-specific antibodies using the hemagglutination inhibition assay. This finding contrasts with recent global reports of H5N1 spillover into cattle populations, particularly in North America, where dairy cows were identified as a novel host in 2024 (5, 6). although our results align with Lang et al.,(7) study which reported that none of tested samples were positive for H5 infection by HI. The absence of detectable antibodies in Iranian cattle suggests either a lack of exposure to H5 AIVs or virus circulation at levels below the detection limit.

Recent studies in other regions have documented H5 AIV infections in cattle. North America's 2024–2025 H5N1 outbreak initially detected the virus in dairy cows, followed by human infections linked to occupational exposure. Similarly, serological surveys in wildlife have identified H5 antibody prevalence as high as 78.7% in certain bird species, underscoring the virus's broad host range (8). An outbreak of H5N1 in dairy cattle in the USA in 2024 in the state of Texas caused clinical signs such as low milk production and depression, The virus was shedding in the milk, which raises a zoonotic concern and inter- or intra-species transmission.(6) despite many interventions, these and related viruses have rapidly spread to infect more than 900 farms in 16 US states The virus has also spread to other animals and is associated with severe and even lethal disease in wild mammals such as foxes, bears, seals, and sea lions; in domesticated cats and dogs; in farmed mink and foxes; and in other livestock, such as goats(9). Concern is mounting that the epizootic will not be contained with traditional farm biosecurity interventions alone and cattle vaccines against H5N1 are urgently needed. In contrast, our findings align with limited prior evidence of H5 exposure in cattle, such as a 2023 study in Europe reporting low seroprevalence (1.1%) in European cattle farms. However, the complete absence of antibodies in this study could highlight potential regional differences in virus ecology or biosecurity practices(10).

The novel clade 2.3.4.4b HPAI reassortant of the H5N1 subtype emerged in 2020, demonstrating unprecedented host adaptability and geographic spread and leading to a dramatic change in disease ecology. This reassortant has disseminated widely and is now found in all regions except Oceania. In early 2024, clade 2.3.4.4b HPAI (H5N1 subtype, genotype B3.13) was identified as the cause of a novel syndrome in lactating dairy cattle in the US is characterized by a severe drop in milk production, abnormal thickened milk (described as 'colostrum-like'), and accompanying non-

specific systemic illness, followed by sustained cow-to-cow transmission in more than 17 US states [4, 10]. Experimental infections in Holstein heifers and lactating cows confirmed viral replication and clinical disease, with high levels of viral RNA detected in milk [10]. Cattle oral tissues support H5N1 virus binding and replication, and virus replicating in the mouth of cattle can be transmitted to the mammary glands of dairy cattle during sucking. Cells in the soft palate, tonsils, root of the tongue, sublingual glands, submandibular glands, lungs, teats, and mammary glands of cattle express both avian-type and human-type sialic acid receptors [11]. The virus shows distinct tropism for mammary gland epithelial cells and efficient cow-to-cow transmission [4]. In the mammary glands of cattle, receptors are highly expressed, facilitating attachment of circulating avian H5 viruses, which might explain the high viral concentrations in milk and support transmission (11). Reports indicate that new herd infections in the US did not involve movement of live cows, and in those cases, spread was presumably through contaminated equipment such as trailers or potentially visitors. Current genomic and epidemiological evidence do not support that wild or peri-domestic birds are spreading HPAI between dairy herds. However, based on experience with HPAI in poultry(12), it is assumed that birds and other animals (including domestic pets, other livestock, and peri-domestic wildlife or feral animals) may act as fomites in transferring virus between cows either within a herd or between premises, highlighting the importance of unwavering vigilance and adherence to biosecurity principles (13). No evidence for H5N1 infection was found in a comprehensive survey in cattle and farm workers in Pakistan 2024. Assuming that samples were representative of cattle and farm workers in Punjab province during the study time period, H5N1 did not appear to be endemic in Punjab cattle population. These findings could also be linked to strict biosecurity measures at those farms. They suggested the inclusion of more cattle farms in the sampling plan in other provinces of Pakistan(12)

Though a gold-standard method for influenza serology, the HI test may lack sensitivity for detecting low-titer antibodies or cross-reactive responses. Studies comparing HI with ELISA have noted that ELISA can enhance the detection of H5/H7 low-pathogenic AIV antibodies in poultry. A retrospective study in cattle from 2023 to late 2024 showed 33.3% positivity for Influenza A viruses NP protein by ELISA these positive samples were further investigated by HI test and positive results were included human and/or swine seasonal viruses but not H5N1 avian Influenza(7). Using a ≥1:16 titer cutoff in this study, as recommended by the World Organization for Animal Health (WOAH), ensures specificity but may overlook subclinical or transient infections. Future studies are needed to investigate integrating ELISA or virus neutralization tests to improve diagnostic accuracy. The detection of H5N1 in dairy cows in North America and sporadic human cases underscores the zoonotic potential of H5 AIVs. While this study found no evidence of cattle exposure in Iran, the global emergence of H5 in new hosts necessitates heightened surveillance. Cattle may act as intermediate hosts, facilitating viral adaptation to mammals, and asymptomatic infections could evade detection. Our results emphasize the need for ongoing monitoring, particularly in regions like Iran, in proximity to wild bird migration routes and poultry farming hubs. This study's limitations include its cross-sectional design, which precludes assessment of temporal trends, and reliance on a single serological method. The 2024–

2025 sampling period coincided with heightened global H5N1 activity, yet no seropositivity was observed. Future research should incorporate longitudinal sampling, broader geographic coverage, and molecular testing (e.g., RT-PCR) to detect active infections. Proactive disease mitigation requires early detection, rapid response, and enhanced biosecurity both on and between farms. These considerations include: 1)Epidemiological assessment: A clinical case definition and a surveillance definition should be established.2) Quarantine and biosecurity: quarantine of infected premises and the establishment of declared areas could aid in mitigating spread via lactating dairy cattle and raw milk.3) Movement controls and testing: In the event of an animal disease outbreak, it's important to have established procedures for safely moving live animals. These procedures need to be carefully considered to prevent further spread of the disease.4) Tracing and surveillance: Clear reporting and enhanced producer awareness support passive surveillance, an essential component for early detection.5) Treatment of infected animals, animal product disposal, and decontamination should target areas and fomites (e.g., footwear, clothing, milking equipment) potentially contaminated with raw milk and wild animal management.

Conclusion

While this study provides critical baseline data for H5 prevalence in two central provinces in Iran, the absence of H5 antibodies in cattle contrasts with global trends. These findings highlight the importance of context-specific surveillance and adaptive risk assessment frameworks to mitigate emerging zoonotic threats. Continued collaboration between the veterinary and public health sectors remains essential to address the evolving epidemiology of H5 AIVs at the human-animal interface.

Conflict of interest

Hereby all authors declare that there is no conflict of Interest.

Ethics

The Institutional Animal Care and Ethics Committee of [Faculty of Veterinary Medicine University of Tehran] examined and approved the study in compliance with the ethical standards of animal research. Before sample collection, informed consent was obtained from the farms' owners; all the procedures adhered to national and international guidelines on animal welfare.

Data availability

All data analysis is available upon request from the corresponding author**.**

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