Title

Insights from a single outbreak: cow-level risk factors associated with HPAI H5N1 clinical disease in lactating Holstein cows

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ABSTRACT

Highly Pathogenic Avian Influenza (**HPAI**) H5N1 (clade 2.3.4.4b) has spillover into dairy cattle populations in the US. A year after the initial outbreak, many uncertainties remain about the virus's epidemiology. This study aims to examine cow-level factors such as days in milk (**DIM**), milk yield, parity, pregnancy, and days carrying calf (**DCC**) that may influence cow`s susceptibility to manifest clinical signs during a HPAI H5N1 outbreak using on-farm herd records. The aims were: (1) to describe the main characteristics of cows manifesting clinical signs, (2) to evaluate the relationship between pregnancy and the manifestation of clinical signs, and (3) to identify other risk factors associated with the manifestation of clinical signs in pregnant cows.

The study was performed in a commercial dairy farm (Colorado, US) that operates in two different locations moving cows between them based on the lactation stage. Due to data availability, the study included only animals from one location, focusing on mid-to-late lactation Holstein cows, most of which were pregnant and over 200 DIM. Cows were housed in 12 pens. Based on the distribution of measured cow-level factors on May 1, 2024, five pens were considered comparable, as they housed groups of cows with similar characteristics within the same facility type. These comparable pens were identified using ANOVA or Chi-Square Test of Independence, post-hoc tests, and network-based clustering.

The location experienced an H5N1 HPAI outbreak, peaking in late May 2024. The study population included all cows present in the herd on May 1, 2024, excluding those in the hospital pen (n = 3,281). Cows were classified as clinical cases if they had "FLU" recorded in their health records by August 31, 2024. The classification followed the outbreak response strategy, which

involved treating only cows with clinical signs like reduced milk production, colostrum-like milk, severe dehydration, and anorexia while keeping records of treated animals.

To describe the characteristics of clinical cases (Aim 1), the proportion of cases across different strata was obtained using the 3,281 cows. T-test was used to compare mean differences in continuous variables (DIM, milk yield, and DCC) between cases and non-cases, while the Chi-Square Test of Independence was applied to assess differences in case status across categorical variables (parity, and pregnancy). To evaluate the relationship between clinical disease and pregnancy (Aim 2), a 1:1 matched cohort of pregnant and non-pregnant cows ($n = 196$), matched by parity, lactation stage, milk yield, and pen ID, was analyzed using conditional logistic regression, with case status as the outcome. For Aim 3, risk factors for clinical disease in pregnant cows were identified by analyzing all pregnant cows from comparable pens ($n = 1,546$) using mixed-effects logistic regression, with case status as the outcome and pen ID as the random effect.

Aim 1 showed an overall proportion of clinical cases of 14.0% ($n = 458$). Significant differences were found for pregnancy (6% in non-pregnant cows vs. 15% in pregnant cows; $p < 0.001$) and parity (9% in 1st parity cows, 16% in 2nd and 3rd parity cows, and 18% in cows with ≥4 parities; $p < 0.001$). Among all 12 pens, the proportion of cases ranged from 7% to 27%, and when restricted to comparable pens ($n = 5$), the variation remained (11% to 24%). For Aim 2, the matched OR, using non-pregnant cows as the reference group, was 4.9 (95% CI: 1.6–14.9). For Aim 3, the OR for multiparous cows vs. primiparous cows was 2.1 (95% CI: 1.5–2.8).

The findings suggest that cow-level factors (pregnancy and parity) and pen-level factors might contribute to HPAI H5N1 clinical disease. Expanding the number of herds studied is critical for a better understanding of these risk factors.

INTRODUCTION

The highly pathogenic avian influenza (**HPAI**) virus H5N1 clade 2.3.4.4b was first detected in the United States in 2021 after its widespread dissemination across Asia and Europe (Bevin et al., 2021). Since its introduction, the virus has caused important outbreaks in wild birds and has had devastating effects on the poultry industry (APHIS-USDA, 2025). In March 2024, an investigation into severe unexplained milk production losses on a Texas dairy farm led to the first confirmed spillover of HPAI H5N1 to dairy cattle (Baker et al., 2024). Molecular assays conducted by the United States Department of Agriculture (**USDA**) confirmed that the clinical signs observed in the affected herd were caused by a spillover event from birds to cattle (Nguyen et al., 2024), marking an unprecedented cross-species transmission. As of March 12, 2025, HPAI H5N1 outbreaks in dairy cattle have affected 985 herds across 17 states (APHIS-USDA, 2025).

The mechanisms of HPAI H5N1 introduction into dairy herds remain unclear. Epidemiological evidence suggests that interstate movement of cattle from affected farms has contributed to disease transmission (Nguyen et al., 2024; Caserta et al., 2024). Additionally, it is hypothesized that farm-to-farm transmission is occurring through everyday activities, such as people, vehicles, and equipment moving between affected and unaffected farms (APHIS-USDA, 2025). However, the spread has been rapid, and other factors may also play a role. Further investigation is needed.

Once introduced into a herd, the primary cow-to-cow transmission routes remain under investigation. While influenza A viruses are primarily respiratory pathogens, the extent of direct or indirect respiratory transmission in dairy cattle is still unclear (Butt et al., 2024). HPAI H5N1 also has a strong affinity for mammary tissue, and experimental studies have shown that intramammary inoculation leads to clinical infection (Burrough et al., 2024; Caserta et al., 2024;

Baker et al., 2024 [unpublished data]; Halwe et al., 2024 [unpublished data]). It has been suggested that contaminated milking equipment, bedding, or direct contact with infected milk could facilitate cow-to-cow transmission, though the role of contaminated environments, feed, and water remains uncertain (Butt et al., 2024).

Dairy cows infected with HPAI H5N1 exhibit a wide range of clinical signs, including abnormal thick, colostrum-like milk, marked milk production loss, dehydration, nasal discharge, lethargy, and gastrointestinal symptoms such as tacky or loose feces (Caserta et al., 2024; Burrough et al., 2024; Oguzie et al., 2024). The severity of the disease varies widely among affected herds, with reported ranges of 3% to 20% (Caserta et al., 2024), 5% to 15% (Rodríguez et al., 2024), and 11% to 14% (Burrough et al., 2024). However, personal communication with dairy producers in California suggests that the percentage of affected animals per farm may be higher. It remains unclear whether cow-level factors influence susceptibility to clinical disease. Identifying cow-level risk factors could provide valuable insights into disease susceptibility and support targeted management and biosecurity strategies to mitigate HPAI H5N1 impact in dairy cattle.

This study aims to examine cow-level factors such as days in milk (**DIM**), milk yield, parity, pregnancy, and days carrying calf (**DCC**) that may influence cow`s susceptibility to manifest clinical signs during a HPAI H5N1 outbreak using on-farm herd records. The aims were: (1) to describe the main characteristics of cows manifesting clinical signs, (2) to evaluate the relationship between pregnancy and the manifestation of clinical signs, and (3) to identify other risk factors associated with the manifestation of clinical signs in pregnant cows.

MATERIALS AND METHODS

Data collection

Herd records from a commercial dairy farm in Colorado (US) were obtained through DairyComp305 (Valley Agricultural Software, Tulare, CA) for this study. The farm experienced an H5N1 HPAI outbreak, with the peak of cows manifesting clinical signs in the last week of May 2024. The farm operated two nearby locations (<3.5 km apart), housing a total of over 7,000 milking cows. Cows were moved between locations based on pregnancy and lactation stage. Location 1 housed early-lactation cows, while Location 2 housed mid-to-late-lactation cows.

Only cows from Location 2 were included in the study. The study population (n = 3,281) consisted of lactating Holstein cows present at this location as of May 1, 2024 (assumed to be before the outbreak was detected), excluding those in the hospital pen. Cows were housed in 12 pens, including 7 free-stall pens (indoor housing with individual stalls; n = 2,126) and 5 open-lot pens (outdoor housing with shared open space; n = 1,155).

The outbreak response was led by the herd veterinarian, focused on three main strategies: (1) minimizing changes to daily farm routines, (2) treating only cows showing clinical signs (reported signs included decline in milk production, colostrum-like milk, severe dehydration, and anorexia), and (3) keeping records of treated cows. To record data, a new item called "*FLU*", without an associated date, was added to the herd management software. Cows were classified as clinical cases if they had "*FLU*" recorded in their health records by August 31, 2024.

Cow-level factors, including parity, DIM, milk yield, pregnancy, and DCC, were also obtained from DairyComp305 (Valley Agricultural Software, Tulare, CA). Additionally, a pen-level factor, pen ID, was included (Table 1). All factors were collected on May 1st, 2024.

Statical analysis

The statistical analysis was performed using the R language for statistical programming (v4.3.2; https://www.r-project.org). The significance level was declared at 0.05.

Identifying comparable pens based on cow-level factors and facility type. Cows were housed in different pens, with their distribution being non-random. Based on measured cow-level factors (parity, DIM, milk yield, pregnancy, and DCC) on May 1, 2024, and facility type (free-stall or open-lot), pens were classified as comparable if they housed groups of cows with similar characteristics within the same facility type. Differences across pens for these factors were identified using ANOVA for continuous variables (DIM, milk yield, and DCC) and the Chi-Square Test of Independence for categorical variables (parity and pregnancy). Post-hoc pairwise comparisons were then performed using Tukey's HSD test for continuous variables and the Chi-Square Test with Bonferroni correction for categorical variables. Finally, a network-based clustering approach was applied, treating pens as nodes and creating edges between pens with non-significant post-hoc comparisons. The analysis was conducted using the *rcompanion* and *igraph* packages in R (Csárdi et al., 2025; Mangiafico, 2025).

Aim 1: Describing the main characteristics of clinical cases. Disease frequency was measured as the proportion of clinical cases. The numerator included cows identified as clinical cases (cows present in the herd on May 1 that had "*FLU*" recorded in their health records by August 31, 2024). The denominator comprised all cows, regardless of case status (cows present in the herd on May 1, 2024). Differences in parity, DIM, milk yield, pregnancy, DCC, and across pens based on case status were explored using unadjusted univariate associations in the entire study population. For cow-level factors, a T-test compared mean differences in continuous variables (DIM, milk yield, and DCC) between cases and non-cases, while the Chi-Square Test of Independence assessed differences in the proportion of clinical cases across categorical variables (parity and pregnancy). For differences across pens, the proportion of clinical cases was reported descriptively for each pen, as the limited number of pens limited statistical inference.

Aim 2: Evaluating the relationship between clinical disease and pregnancy. A 1:1 matched subset was created by pairing pregnant and non-pregnant cows based on parity, lactation stage (100–200 DIM, 200–300 DIM, >300 DIM), milk yield (<20 L/d, 20–30 L/d, 30–40 L/d, >40 L/d), and pen ID. Only pregnant cows that conceived before 150 DIM and non-pregnant cows that were between 128 and 375 DIM were included. The statistical model used was conditional logistic regression (*Epi* package; Carstensen et al., 2024), with case status as the outcome and pregnancy as the predictor. The measure of association was the matched odds ratio (**OR**). Additionally, the proportion of clinical cases was reported for pregnant and non-pregnant cows.

Aim 3: Identifying risk factors in pregnant cows. A subset of the study population was created based on the inclusion criteria of being a pregnant cow from a pen previously identified as comparable. Non-pregnant cows from these pens and all cows in other pens were excluded. The statistical model used was mixed-effects logistic regression (*lme4* package; Bates et al., 2015), with pen ID as a random effect and case status as the outcome. The fixed effects included parity, DIM, DCC, and milk yield. Since the continuous variables did not meet the linearity assumption of the logit, they were categorized based on industry standards. Linearity of the logit was visually assessed by plotting the continuous predictors, divided into equal-sized bins, against their corresponding log-odds of the outcome. DIM was classified as mid-lactation (100–200 DIM), late lactation (200–300 DIM), and extended lactation (>300 DIM). DCC was categorized as early gestation (<90 DCC), mid-gestation (90–180 DCC), and late gestation (>180 DCC).

Milk yield was grouped into low producers (<75% of the herd average), average producers, and high producers (>125% of the herd average). Additionally, cows with two or more parities were grouped into a single category. The model building strategy consisted of an initial variable screening with a significance threshold of 0.2, followed by backward selection, considering both statistical significance and a 20% change-in-estimate criterion. The measure of association was the OR. Additionally, the proportion of clinical cases was also reported for the factors included in the final model.

RESULTS

Based on the herd as of May 1, 2024, the study population consisted of 3,281 cows, the majority of which were over 200 DIM (75.0%) and pregnant (90.6%), with DCC ranging from 60 to 225 days. By August 31, 2024, 458 (14.0%) of them were identified as clinical cases. This population was used to describe the main characteristics of clinical cases (**Aim 1**). The characteristics of cases, non-cases, and the overall study population are summarized in **Table 2**. No significant differences were observed between cases and non-cases for DIM ($p = 0.15$), DCC ($p = 0.42$), and milk yield ($p = 0.39$). However, pregnancy ($p < 0.001$) and parity ($p < 0.001$) showed significant differences. The proportion of clinical cases was 6% in non-pregnant cows and 15% in pregnant cows. Similarly, it was 9% in 1st parity cows, increasing to 16% in 2nd and 3rd parity cows and 18% in cows with 4 or more parities. Across all 12 pens, the proportion of clinical cases varied, ranging from 7% to 27%. Among them, 5 pens were identified as comparable based on cow-level factors and facility type. When restricting the analysis to these comparable pens, the differences remained, ranging from 11% to 24%. No p-values were calculated. (**Fig. 1**).

The matched subset for evaluating the relationship between clinical disease and pregnancy (**Aim 2**) included 196 cows (98 pregnant, 98 non-pregnant) with an average parity of 2.5, 266 DIM, and 167 DCC (**Table 2**). The observed proportion of clinical cases was 4.1% in non-pregnant cows and 18.4% in pregnant cows. The matched OR, using non-pregnant cows as the reference group, was 4.9 (95% CI: 1.6–14.9) (**Fig. 2A**).

Table 2 also describes the subset of pregnant cows (n = 1,556) used to identify risk factors in this group (**Aim 3**). The best-fitting model included parity, categorized as primiparous vs. multiparous, as the only predictor. The observed proportion of clinical cases was 9.0% in primiparous cows and 18.0% in cows with two or more parities. The OR, using 1st parity cows as the reference group, was 2.1 (95% CI: 1.5–2.8) (**Fig. 2B**).

DISCUSSION

A year after the first official case of HPAI H5N1 in dairy cows (Texas, US; March 24, 2024), many uncertainties remain about the disease, including within-farm risk factors that influence the manifestation of clinical signs. This study investigated cow-level factors that may increase the risk of clinical disease during an outbreak on a Colorado dairy farm. Although multiple factors were analyzed, only parity and pregnancy appeared to influence clinical disease. However, these findings are based on a single outbreak, limiting their external validity and generalizability.

The measure of disease frequency used in this study was the proportion of clinical cases. While this provides valuable insights into disease occurrence, it does not represent a true incidence measure due to the lack of follow-up time and a herd composition change of more than 10% between May 1, 2024, and August 31, 2024. The authors considered the OR more appropriate than the risk ratio (**RR**) as a measure of association to prevent the use of an inaccurate incidence. Since the overall proportion of clinical cases was 14.0%, suggesting that the disease is not rare, the OR may overestimate the RR. However, the direction of the association should not vary (Dohoo et al., 2012). This justifies the authors' decision to use logistic regression.

Cows were classified as clinical cases if they had "*FLU*" recorded in their health records. The classification followed the outbreak response strategy, which involved treating only cows with clinical signs such as reduced milk production, colostrum-like milk, severe dehydration, and anorexia while maintaining records of treated animals. Results should be interpreted with consideration of the subjectivity of case definition and potential changes in criteria over time. The clinical signs reported by the study farm align with those described by Caserta et al. (2024) in nine different dairy farms, which included reduced feed intake, anorexia, depression, dehydration, respiratory symptoms (nasal discharge, increased respiratory rate, abnormal lung sounds), gastrointestinal symptoms (diarrhea, constipation, abnormal fecal consistency, tacky manure), abrupt decreases in milk production, yellowish or creamy milk, increased somatic cell count, mastitis, neurological signs (poor rumen motility, decreased capillary refill time), and increased mortality.

The overall proportion of clinically affected animals in the study population was 14%, falling within the reported range of 3% to 20% (Caserta et al., 2024), 5% to 15% (Rodríguez et al., 2024), and 11% to 14% (Burrough et al., 2024). In addition to the reported between-herd differences, we observed variation between pens. Despite the small number of pens (n = 12), the proportion of clinical cases varied from 7% to 27%. Even among comparable pens (those housing groups of cows with similar characteristics within the same facility type), the differences remained (11% to 24%). It suggests that cow-level, pen-level, or farm-level factors may influence the cow’s susceptibility to manifest clinical signs. Further research is needed.

Burrough et al. (2024) reported that HPAI H5N1 clinical disease was more common in multiparous cows during mid to late lactation.

After controlling for parity, DIM, milk yield, and pen, we observed that pregnant cows had significantly higher odds of manifesting clinical signs compared to non-pregnant cows. In the matched subset, cows had an average of 167 DCC and 266 DIM, meaning this comparison excludes early pregnancy and early lactation and applies to cows in advanced gestation and later lactation. Notably, in commercial dairy operations, most mid-to-late lactation cows are pregnant. We hypothesize that the risk of HPAI H5N1 clinical disease could increase as gestation advances. Pregnant females are generally more vulnerable to viral infections due to immune adaptations that support fetal development but weaken maternal immunity (Creisher and Klein, 2024). Increased progesterone and estrogen levels further contribute to these immune changes (Cervantes et al., 2023). This finding may explain the higher incidence of HPAI H5N1 during mid-to-late lactation reported by Burrough et al. (2024), although other events common at this stage should also be explored.

Burrough et al. (2024) also reported a higher incidence in multiparous cows. We found that multiparous cows had higher odds of manifesting clinical signs than primiparous cows. This increased susceptibility is likely due to immunological, metabolic, and physiological factors (Lean et al., 2023). Older cows show chronic immune activation, with increased expression of immune defense genes, suggesting immunosenescence. This prolonged activation may weaken their response to new pathogens like H5N1. They also experience greater baseline inflammation, which can further compromise immunity (Buggiotti et al., 2021). Metabolic differences also play a role. Primiparous cows rely more on beta-oxidation for energy, while multiparous cows shift toward glycolysis, indicating greater metabolic stress. Combined with declining insulin-like growth factor-1 (IGF-I) levels, this shift affects immune function and increases infection risk (Ruprechter et al., 2020).

CONCLUSION

The findings suggest that cow-level, pen-level, and farm-level factors might contribute to HPAI H5N1 clinical disease. Pregnant cows had significantly higher odds of manifesting clinical signs than non-pregnant cows, and multiparous cows had higher odds than primiparous cows. These results indicate that pregnancy and parity may be important cow-level risk factors. Further investigation is needed. Expanding the number of herds studied is critical to better understanding the risk factors and implementing targeted strategies to mitigate the impact of HPAI H5N1 in dairy herds.

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Table 1. Description of cow-level and pen-level factors collected from DairyComp305 (Valley Agricultural Software, Tulare, CA)

Factor	Description
Flu	Dichotomous variable (YES or NO) indicating whether the cow was identified with clinical disease by farm personnel at any time before August 31, 2024.
Parity	Current lactation number of the cow.
DIM	Number of days since the last calving on May 1, 2024.
Milk yield	7-day average daily milk yield (in liters) as of May 1, 2024.
Pregnancy status	Dichotomous variable (YES or NO) indicating whether the cow was diagnosed as pregnant by May 1, 2024.
DCC	Number of days since conception on May 1, 2024.
Pen ID	Corral where the cow was housed on May 1, 2024.

Table 2. Descriptive statistics for each subset by study aim

Aim 1: Describing the main characteristics of clinical cases	**Total**	**Non-cases**	**Cases**
Cohort (n)	3,281	2823 (86.0%)	458 (14.0%)
Parity	2.3	2.3	2.6
Days in milk	250	250	255
Pregnant cows (%)	90.6	89.7	96.3
Days carrying calf	129	160	163
7d-milk yield (l/d)	33.7	33.7	33.9

Aim 2: Estimating the effect of pregnancy	**Total**	**Non-pregnant**	**Pregnant**
Cohort (n)	196	98 (50.0%)	98 (50.0%)
Parity	2.5	2.5	2.5
Days in milk	266	268	264
Days carrying calf	NA	NA	167
7d-milk yield (l/d)	28.7	29.0	28.4
Cases (%)	11.2	4.1	18.4

Aim 3: Identifying risk factors in pregnant cows	**Total**	**Non-cases**	**Cases**
Cohort (n)	1556	1329 (85.4%)	227 (14.6%)
Parity	2.1	2.1	2.5
Days in milk	251	251	251
Pregnant cows (%)	100.0	100.0	100.0
Days carrying calf	144	144	144
7d-milk yield (l/d)	34.6	34.5	34.7

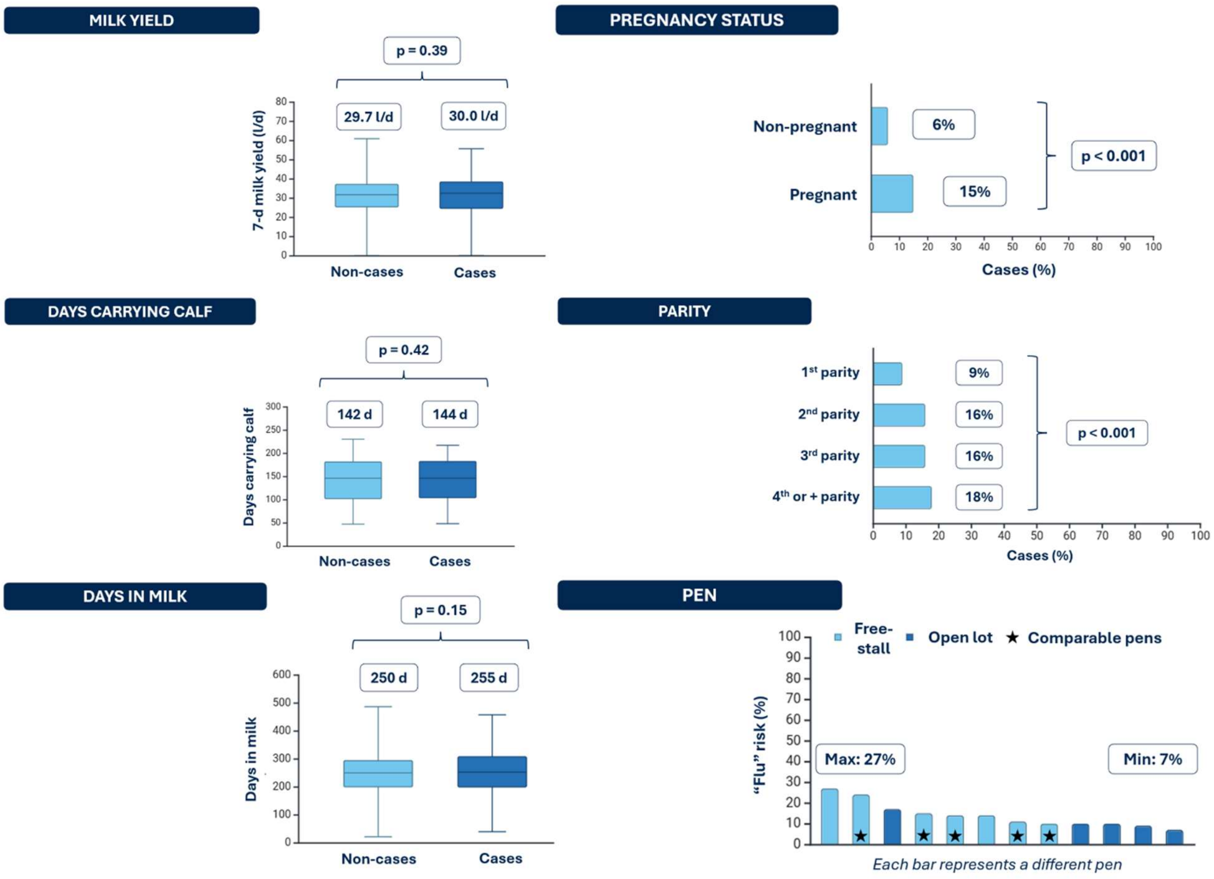

Figure 1. Main characteristics of HPAI H5N1 clinical cases by milk yield, pregnancy, days carrying calf, parity, days in milk, and pen ID (Aim 1).

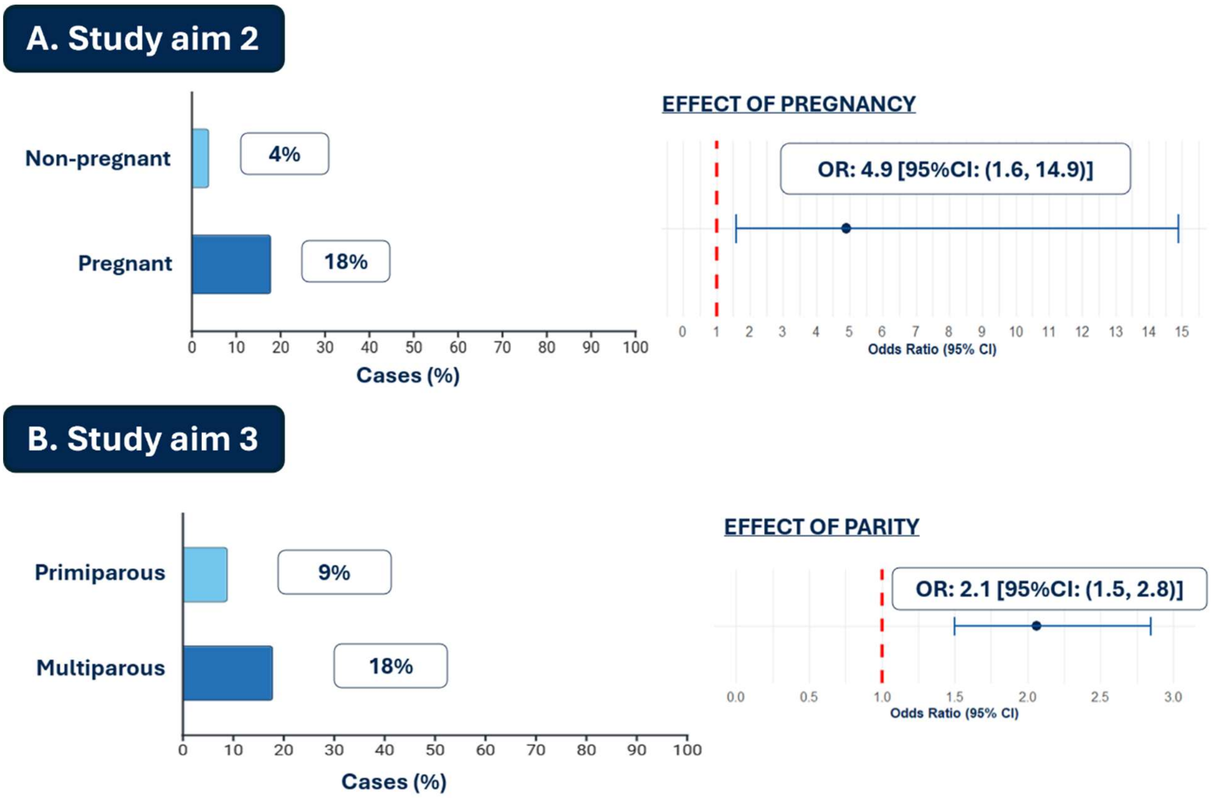

Figure 2. (A) Proportion of clinical cases and matched odds ratio for Aim 2: Estimating the effect of pregnancy; (B) Proportion of clinical cases and odds ratio for Aim 3: Identifying risk factors in pregnant cows.