

## Seroprevalence of *Chlamydia abortus* in Anestrous Cattle of Nawalpur and Chitwan District, Nepal

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### ABSTRACT

*Chlamydia abortus* are gram negative coccoid, obligate intracellular zoonotic bacteria responsible for abortion and other reproductive problems in dairy cattle. This leads to sub-fertility and infertility in cattle thereby causing significant economic losses to dairy farmers. However, the status of *Chlamydia abortus* in Nawalpur and Chitwan districts, which are the dairy pockets of Nepal, are poorly understood. The objective of this study was to determine the seroprevalence of antibodies to *Chlamydia abortus* in anestrous dairy cattle of Nawalpur and Chitwan districts in relatively low risk winter season. In total, 92 cattle serum (Nawalpur (n= 27) and Chitwan (n= 65) samples were collected from April 2018 to May 2019 and tested using a *Chlamydia abortus* specific indirect ELISA kit to evaluate the seropositivity status of cattle. Results showed that only 2 (2.17%) cattle were seropositive for *Chlamydia abortus*. Both the positive cattle were Jerseys from Nawalpur area with a history of abortion. The ages of the seropositive cattle were 3 and 4 years. In conclusion, there was a low seroprevalence of *C. abortus* in dairy cattle of Nawalpur and Chitwan districts. The presence of this bacterium suggests that it is likely to be a cause of unidentified abortion in dairy cattle in Nepal. We suggest screening dairy animals for *C. abortus* infection to minimize this risk.

**Keywords:** Abortion; Cattle; *Chlamydia abortus*; iELISA; Seroprevalence

### INTRODUCTION

In Nepal livestock and its product contribute 11.5 % of national Gross Domestic Product (GDP) (CBS, 2011), and 25.68% of the Agricultural Gross Domestic Product (AGDP). Among 7,302,808 cattle in Nepal, Chitwan and Nawalpur contain 71,864 and 96,113 cattle annually producing 14,947Mt and 18,451Mt milk respectively (MoLD., 2017) (VHLSEC, 2073/74). Nepal embodies dairy cattle production as one of the important contributors to the national economy. Anestrus has major economic consequences for the dairy industry worldwide (Dziuk, 1983). There are various causes of anestrous including nutrition, infectious disease (parasitic, bacterial, viral, and protozoal) and poor management practices. *Chlamydia abortus* is reported to be one of the bacterial causes of anestrous in dairy cattle. Members of the genus *Chlamydia abortus* are Gram negative coccoid, obligate intracellular bacteria within the family *Chlamydiaceae* and order *Chlamydiales*.

*Chlamydia abortus* (*C. abortus*) is endemic in ruminants throughout the world. It can efficiently colonize the placental trophoblasts and is one of the causative agents of abortion and fetal loss in sheep, goats, and cattle in many countries (Shewen, 1980). It is reported to be the causal organism for Epizootic Bovine Abortion (EBA) in milking cows which results in mastitis, reproductive issues such as endometritis, changes in length of open period and vaginitis, pneumonia, conjunctivitis, enteritis, polyarthritis and encephalitis. (Corner, 1968); (Twomey, 2003); (PRAGA-AYALA, 2014). *Chlamydia* can be shed and transmitted by almost all secretions and excretions (vaginal, ocular, and nasal fluids, semen and urine with fecal shedding being the most important route. Due to various pathogenetic peculiarities, chlamydial infections do not necessarily lead to clinical illness causing chronic inflammatory reactions and dysfunctionality of different organs. Subclinical chlamydial infections are probably economically more important than rare outbreaks of severe chlamydial disease. Generally cases become agent negative before they become serologically negative. So, serology is considered to be most important in the diagnosis of latent or chronic infection, as well as in confirming ongoing disease (Shewen, 1980). Infected animals may be a source of infection in humans and lead to severe outcomes including respiratory disorder and miscarriage (Walder, 2005 ); (Ortega, 2015).

Monitoring of this zoonotic pathogen in dairy cattle has become a very important issue in Nepal. Detailed reports of serosurveillance of Chlamydiosis within the national dairy cattle herd have not been conducted yet in Nepal. This research will help to assess the seroprevalence of *Chlamydia abortus* and its impact in causing anestrus in cattle of Nawalpur and Chitwan districts which are a major source of milk for Nepal. Likewise, this research will provide baseline information on the demographics and aetiology of subclinical forms of Chlamydiosis and its consequences for dairy herd fertility. So, the objective of the present study was to determine the prevalence of antibodies to *Chlamydia abortus* in anestrus and aborting dairy cattle in Nawalpur and Chitwan. The study was conducted in winter when the risk of infection is quite lower. The identification of age, breed, parity and previous history of abortion as contributory factors are all considered in the study.

## MATERIALS AND METHODS

A cross sectional descriptive study on anestrus cattle of improved dairy cattle breeds (Jersey cross and Holstein cross) was carried out from November 2018 to May 2019 in Nawalpur and Chitwan districts of Nepal. Sample size was calculated using the given formula (Daniel, 1999).

$$n=Z^2 P (1-P)/d^2 \text{ where}$$

n= sample size,

Z = Z statistic for a level of confidence; at 95% level of confidence and 5% level of significance, Z value is 1.96

P= expected prevalence or proportion (in proportion of one; prevalence of Chlamydiosis in Nawalpur and Chitwan was 2.65% (0.02675). (NCRP, 2017/18)

d = Precision limit or proportion of sampling error which is 5% confidence limit (0.05)

Hence  $n= (1.96)^2 * 0.02675 (1-0.02675) / (0.05)^2 = 40.005$

While the total number of samples collected was 92 (Nawalpur (n=27) and Chitwan (n=65)).

## SAMPLING PROCEDURE

Purposive sampling was used. Blood samples from 92 cattle displaying signs or history of anestrus were collected from the jugular vein and transferred into 3ml clot activators. Samples were transported in icebox to the laboratory of the National Cattle Research Program. On the same day, serum was extracted by centrifugation of the clot activators 3000 rpm for 10 minutes and stored at 4°C until assay.

## ELISA PROCEDURE

*C. abortus*-specific antibodies were determined in the sera according to the manufacturer's instructions. An indirect ELISA commercial kit The IDScreen® *Chlamydophila abortus* Indirect Multispecies ELISA from IDVet® (Montpellier, France) was used according to the instructions of the manufacturer. This assay reported a sensitivity of 100% and specificity of 99.7% (Pourquier, 2007). Briefly, 10µl each of negative control, positive control and serum samples were diluted in 90µl of dilution buffer in 92 well micro titre plates, incubated at 21°C for 45 min and plates finally washed three times by pipetting 300µl Wash Solution onto plates. For the detection of specific antibodies Conjugate 1X was added (100µl/well), plates incubated at 21 °C for 30 min, washed three times with Wash Solution (300µl/well) followed by the addition of the substrate solution (100µl/well) and incubation at 21°C for 15 min. The reaction was stopped by adding 100µl stop solution to each well. Optical densities of the samples were measured using spectrophotometry at a wavelength of 450 nm. Results were given as values (%) and calculated using the measured optical densities (OD) and the equation as follows:

$$\frac{S}{P}(\%) = \frac{OD_{450nm} \text{ test serum}}{(OD_{450nm} \text{ positive control})}$$

Sera with values  $\geq 60\%$  were considered as positive for *Chlamydia abortus* antibodies. Sera with values of 50–60% were considered doubtful and  $\leq 50\%$  were negative.

## ESTIMATION OF TRUE PREVALENCE

If a test with less than 100% sensitivity and specificity is used to estimate prevalence of some characteristic, that estimate will invariably be biased. If the sensitivity and specificity of the test are known and if the apparent prevalence is greater than (specificity – 1), we can estimate the true prevalence with the **Rogan-Gladen estimator**

$$\text{True Prevalence} = \frac{\text{Apparent Prevalence} + (\text{Specificity} - 1)}{\text{Specificity} + (\text{Sensitivity} - 1)}$$

Where,

- The **true prevalence** is the proportion of all those which are tested and are actually positive
- The **apparent prevalence** is the proportion of those which are tested and rightly or wrongly, test positive

The iELISA used to detect *C. abortus*-specific antibodies in the present study had a sensitivity of 100% and specificity a of 99.7% (Pourquier, 2007)

## DATA ANALYSIS

The data analysis was conducted with the help of Microsoft Excel-10 and IBM SPSS Statistics 20 (IBM Corporation 1989, 2011). Statistical analysis of seroprevalence of *Chlamydia abortus* in dairy cattle of 2 districts was performed using IBM Statistics 20. Prevalence was studied among different age groups (less than 2 years, 2-5 years and 5 years and above), parity (1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup> 4<sup>th</sup> and 5<sup>th</sup>), history of abortion (aborted and non-aborted), and breed (Jersey cross and Holstein cross) using a chi-squared test to study the association between *Chlamydia* and different variables. The differences were considered statistically significant if  $P < 0.05$ .

## RESULTS

Out of the total (92) samples (27: Nawalpur and 65: Chitwan) collected from the anestrus dairy cattle, 2.17% (2/92) were positive and 95.65% (88/92) were negative while 2.17% (2/92) yielded doubtful results which means weakly positive according to the kit manual for a *Chlamydia abortus* infection on Indirect Enzyme Linked Immuno-sorbent Assay (iELISA). So, the overall seroprevalence in this study is 2.17% while the overall true prevalence of *C. abortus* in our study is 2.26%

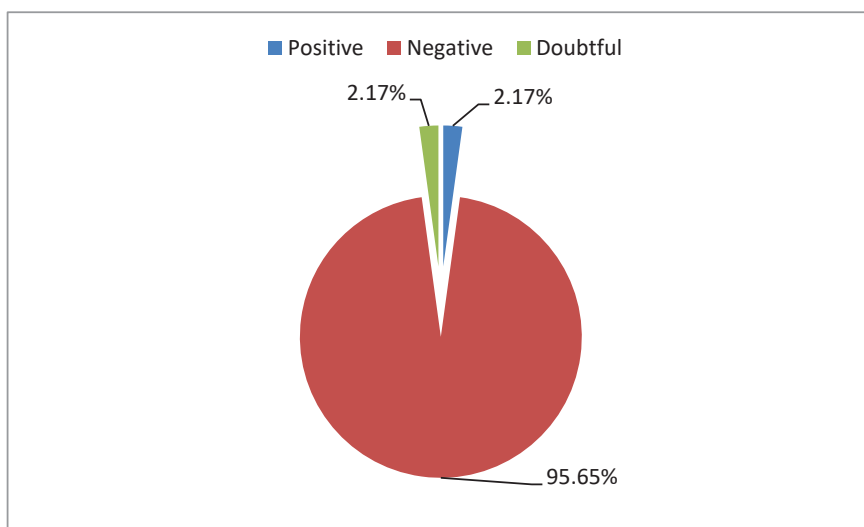


Figure 1: Overall seroprevalence of *Chlamydia abortus* detected in serum from 92 dairy cows in Nepal (27: from district Nawalpur and 65 from district Chitwan).

### District-wise seroprevalence

Among 27 cattle tested from Nawalpur, seroprevalence was (7.40%) and among 65 cattle tested from Chitwan there was no seropositivity. Some (3.08%) of the iELISA tests were inconclusive in samples collected from the District Chitwan. There was no significant difference between the district and seropositivity ( $p=0.058$ ).

Table 1: Chi-square univariate analysis of relationships between location and the detection of chlamydia serum antibodies in serum from 92 dairy cows in Nepal (27 from district Nawalpur and 65 from district Chitwan).

Variable	Number of animals	Serum antibodies to <i>C. abortus</i>				P value
		Positive	Negative	Doubtful	Prevalence rate (%)	
District						0.058
Nawalpur	27	2	25	0	7.40	
Chitwan	65	0	63	2	0	

### Age wise seroprevalence

No seroprevalence of *Chlamydia abortus* was found in dairy cattle less than 2 years old while one test was inconclusive (11.11%). Similarly, no seroprevalence was found in the age group above 5 years and above while one test was inconclusive (1/44; 2.27%). Seroprevalence was highest in the 2-5 years age group at 4.08% (2/39), although this agewise difference in seroprevalence was not significant ( $p=0.139$ ).

Table 2: Chi-square univariate analysis of relationships between age and chlamydia serum antibodies detected in serum from 92 dairy cows in Nepal (27: from district Nawalpur and 65 from district Chitwan).

Variables	Number of animals	Serum antibody to <i>C. abortus</i>				P value
		Positive	Negative	Doubtful	Prevalence (%)	
Age of animals						
<2 years	9	0	8	1	0	0.139
2-5 years	39	2	37	0	4.08	
≥5 years	44	0	43	1	0	

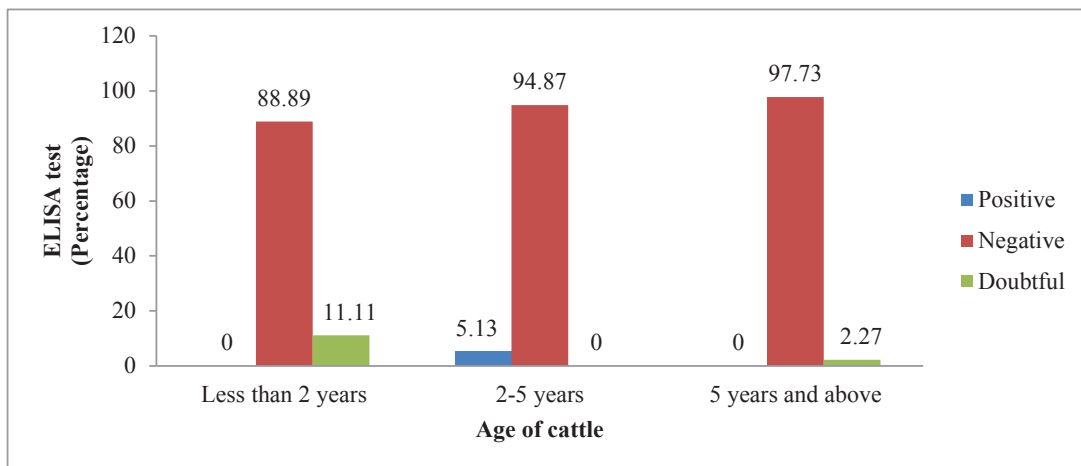


Figure 2: Age wise seroprevalence of *C. abortus* by ELISA test detected in serum from 92 dairy cows in Nepal (27: from district Nawalpur and 65 from district Chitwan).

### Abortion wise seroprevalence

Seroprevalence of *Chlamydia abortus* in cattle with a recent history of abortion was 11.11% while cattle with a normal calving and no abortions, none of them gave a positive result. Inconclusive results were observed in 2.70% (2/74) of the non-aborted cattle. The result showed that there was a significant difference in the prevalence ( $p=0.012$ ).

Table 3: Chi-square univariate analysis of relationships between history of abortion and the expression of chlamydia serum antibodies in serum from 92 dairy cows in Nepal (27: from district Nawalpur and 65 from district Chitwan).

Variable	N	Serum antibody to <i>C. abortus</i>			Prevalence (%)	P value
		Positive	Negative	Doubtful		
Aborted	18	2	16	0	11.11	0.012*
Non-aborted	74	0	72	2	0	*= $p<0.05$

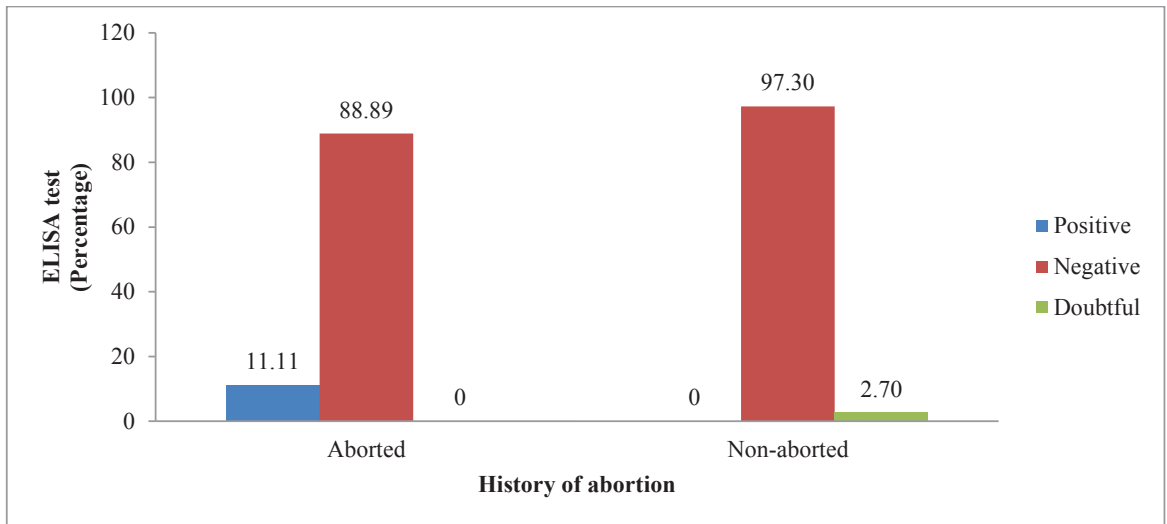


Figure 3: The relationship between the incidence of abortion and seroprevalence of *C. abortus* by ELISA test in serum from 92 dairy cows in Nepal (27: from district Nawalpur and 65 from district Chitwan).

### Breed wise seroprevalence

Out of the total of 92 cattle tested, 56 were Jersey cross and 36 were Holstein cross. Out of these seroprevalence in Jersey cross breed was found to be 3.57% while 3.57% yielded an inclusive result. Seroprevalence was completely negative in the Holstein cross breed but there was no significant difference in the prevalence ( $p=0.261$ ).

Table 4: Chi-square univariate analysis of relationships between breed and chlamydia serum antibodies detected in serum from 92 dairy cows in Nepal (27: from district Nawalpur and 65 from district Chitwan).

Variable	n	Serum antibody to <i>C. abortus</i>			Prevalence (%)	P value
		Positive	Negative	Doubtful		
Jersey cross	56	2	52	2	3.57	0.261
Holstein cross	36	0	36	0	0	

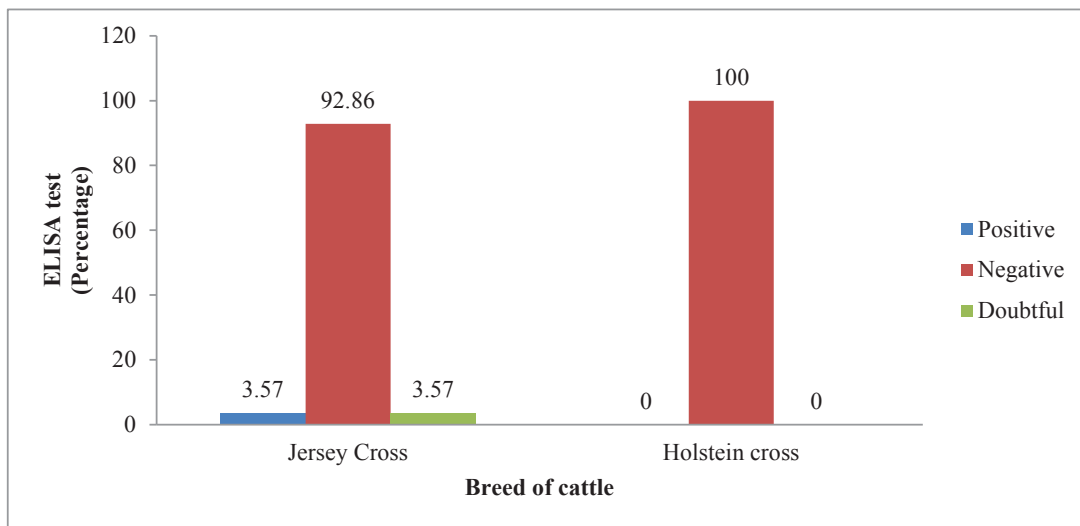


Figure 4: Breed wise seroprevalence of *C. abortus* by ELISA test in serum from 92 dairy cows in Nepal (27: from district Nawalpur and 65 from district Chitwan).

**Method of insemination wise**

Out of the total of 92 cattle, 27 were serviced naturally and the remaining 65 were artificially inseminated. Seroprevalence was highest (7.40%) in naturally serviced cattle while none of the artificially inseminated cattle were seropositive to *Chlamydia abortus*. Of the artificially inseminated cattle 3.08% gave an inconclusive result to *C. abortus* antibody detection. The result showed that there was no significant difference between the two types of service used (p=0.058) Table 5: Chi-square univariate analysis of relationships between type of service used and chlamydia serum antibody expression from 92 dairy cows in Nepal (27: from district Nawalpur and 65 from district Chitwan).

Variable	n	Serum antibody to <i>C. abortus</i>			Prevalence (%)	P value
		Positive	Negative	Doubtful		
Artificial	65	0	63	2	0	0.058
Natural	27	2	25	0	7.40	



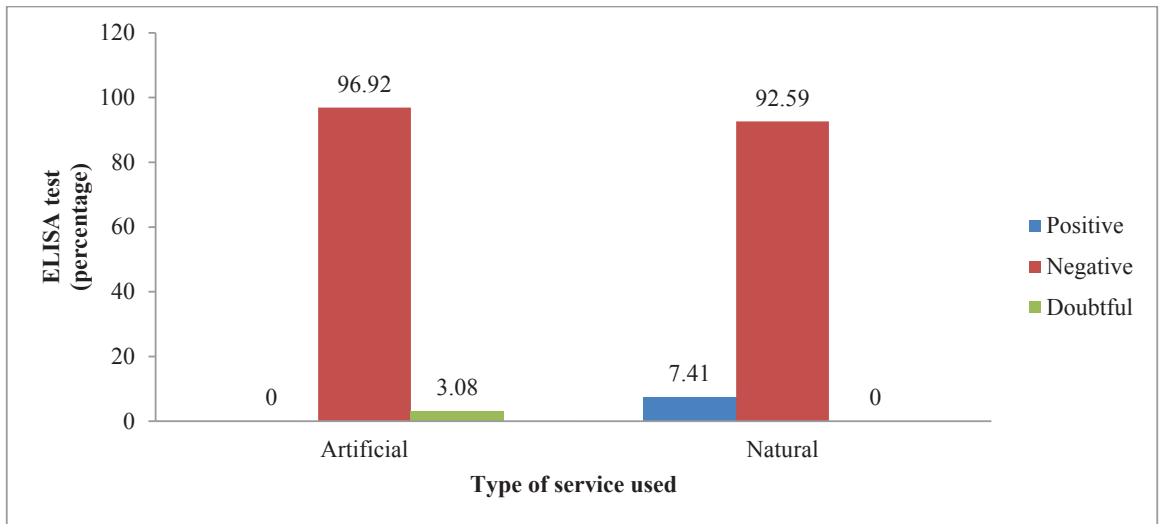


Figure 5: Seroprevalence of *C. abortus* according to type of service used detected in 92 dairy cows in Nepal (27: from district Nawalpur and 65 from district Chitwan).

## DISCUSSION

The overall prevalence rate obtained from the present study exhibited a low seroprevalence (2.7%) of *C. abortus*. This suggests that this bacterial species is rarely present in dairy cattle of Nepal. A similar study conducted in Nepal by National Cattle Research Program in 2017/18 (NCRP, 2017/18) at the same study site using similar methodology has reported the overall seroprevalence of 2.65% which was slightly higher than reported here. The previous study was conducted in spring while our study was conducted in the early winter season. The disease is most commonly diagnosed in the lambing season during late winter or early spring (Essig, 2015). No evidence of vaccination against *Chlamydia abortus* in Nepal shows that seroprevalence has not been altered in association with the use of vaccination. However, occurrence of seropositive animals suggests previous contact with *C. abortus*, especially in herds in the district of Nawalpur. It has been reported that prevalence is associated with large mean herd size (Jiménez-Estrada, 2008). So, the low prevalence in our study area may be associated with the smaller mean herd size using different rearing strategies in a zero grazing system throughout the rearing period. Out of a total 92 samples tested (27: Nawalpur and 65: Chitwan), only 2 (2.17%) were positive and both of them were from Nawalpur. But the differences were not significant ( $p < 0.05$ ) owing to the larger mean herd size in Nawalpur relative to that of Chitwan. Infected sheep and goats act as chronic carriers of *C. abortus* persistently shedding them in faeces, urine, uterine and vaginal discharge. None of the dairy cattle herds in the study area had close contact with sheep and other small ruminants to have enabled cross species transmission. In studies conducted in trachoma endemic regions in Nepal, trachoma was diagnosed in 63% of samples (serum and ocular swabs), collected from humans. These were the result of single or mixed infections caused by 5 zoonotic species of chlamydia; *C. trachomatis*, *C. psittaci*, *C. pecorum*, *C. suis* and *C. pneumonia*. Here, none of the samples yielded positive results for *C. abortus* where the test was conducted by detected by DNA Microarray Assay and by quantitative Real-Time PCR (Dean, 2013). This shows the lower prevalence of *C. abortus* species as a zoonotic pathogen in Nepal.

The iELISA used in the present study uses a synthetic antigen from a major outer-membrane protein (Momp) specific to *Chlamydia abortus*, which has high sensitivity (100%) and specificity (99.7%). This test reduces the frequency of non-specific reactions (Pourquier, 2007). It is a powerful tool to test both suspected *C. abortus* infected and healthy herds. Our findings here are consistent with a similar study carried out in Mexico by (Praga-ayala, 2014) where the total seroprevalence was found to be 0.73% using iELISA. The lower seroprevalence in Mexico was explained by lower interactions with other herds and particularly ovine herds and wild ruminants which exhibit a higher prevalence of *Chlamydia abortus*. The higher prevalence (80.7%) in placental samples by CFT in India reported by (Nanda, 1992) compared to our iELISA result can be explained by the fact that a bacteriologically positive result does not necessarily mean that the individual has developed antibodies. The active agent in the ELISA has a high affinity for reproductive tissues (Sachse K, 2009). Antigenic cross-reactivity between *C. abortus* and *C. pecorum*, which is endemic in small ruminants, as well as with some Gram-negative bacteria (e.g. *Acinetobacter*), can give rise to false-positive CF test results (OIE, 2018). A similar study conducted in Sweden among the dairy cows with reproductive disorders using rELISA showed a seroprevalence of 0.4% (Godin, 2008). The possible confounding factors for lower seroprevalence are more or less similar to our study. They are self-contained herds, little interactions with other species, little movement of animals and lower seroprevalence among the ovine population of Sweden. There are no published data on ovine seroprevalence in Nepal. We have identified a significant association ( $p=0.01$ ) of seroprevalence of *Chlamydia abortus* with abortion in our study. Similarly there was a significant relationship between the presence of serum antibodies and a preceding abortion ( $P < 0.001$ ) in a study carried out by (Wehrend, 2005) in dairy herds with fertility disorders in Hessen (Germany) using a genus specific ELISA. In our study prevalence of *Chlamydia abortus* was restricted to the Jersey cross cows and was not found in other breeds like Holstein Friesian crosses, local and pure breeds. This might be due to the larger sample size of the Jersey cross breed and a higher incidence of reproductive issues like mastitis, abortion and repeat breeding, among these Jersey cross cows. In a study carried out in Germany by (Kauffold J. H., 2007) prevalence of *Chlamydia abortus* was 2.5% (3/120) as detected by PCR in semen samples was reported indicating excretion of the organism in semen and possibly transmission as a venereal disease. Similarly, a higher seroprevalence (7.41%) compared to artificially inseminated cattle was found among naturally bred cattle in our study. Although the result was not significant, it might suggest possible venereal transmission from breeding bulls to cows. But prevalence of *Chlamydia abortus* among breeding bulls in Nepal is yet to be investigated.

The inconclusive results achieved by iELISA for infection with *Chlamydia abortus* may be related to a number of factors. Such results might have resulted from cross-reactivity with related antibodies using this test kit. Use of this kit has been reported to reduce non-specific reactions without eliminating them. Thus we could only test 92 serum samples. *Chlamydia abortus* is more prevalent in early spring to early summer while our study was conducted in early winter season when the risk of exposure was relatively lower. Likewise, we conducted our study for a month only while a more effective program detection would involve seromonitoring twice a year in the early and late monsoon seasons. Similarly the use of PCR technology would have provided more definitive results, however their cost prevented their use in the present study.

Since no vaccination is practiced in the study area, the seroprevalence observed was due to natural infection. The seroprevalence was higher in Jersey cattle of 3-4 years of age with recent history of

abortion. It also suggests that *Chlamydia abortus* is not related to the anestrous condition in the cattle on our study site. However the incidence of abortion is likely to be associated significantly with seroprevalence of *C. abortus* in our study. The seroprevalence was significantly higher in cattle with a recent history of abortion ( $p < 0.05$ ).

## CONCLUSION

Hence, the study suggests that there is a low seroprevalence of *Chlamydia abortus* in anestrous dairy cattle of Nawalpur and Chitwan districts of Nepal at the time of this study. We suggest that dairy cattle herd to be screened on a routine basis to rule out a possible causal relationship between the incidence of *C. abortus* infection and the prevalence of abortion. The issue would be resolved with the elimination of this pathogen from breeding herds.

## Ethical statement

All animal owners provided their oral consent before the collection of the blood samples and agreed to answer the related survey questions. The cattle were sampled by a qualified veterinarian following all applicable guidelines for the care and use of animal.

## Conflict of interest

The authors declare that they have no conflict of interest.

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