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Leptospira interrogans Serovar *Hardjo* Seroprevalence and Farming Practices on Small-Scale Dairy Farms in North Eastern India; Insights Gained from a Cross-Sectional Study

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Abstract: Leptospirosis is a zoonotic disease of major public health concern in India. Bovines play an important role in maintaining and transmitting this disease and proximity between dairy cows and humans makes the dairy cow-human nexus a transmission route of public health interest, yet one currently under-examined in North Eastern India. We report a cross-sectional survey carried out on small-scale dairy farms in the states of Assam and Bihar in North Eastern India investigating seroprevalence for *Leptospira interrogans* serovar *Hardjo*, the most common pathogenic serovar reported in cattle worldwide. Higher seroprevalence was reported on dairy farms in Bihar 4.5% (95% CI 2.6–7.5%) than in Assam 1.2% (95% CI 0.42–3.6%), but overall seroprevalence levels were low. The study is the first indication of leptospirosis circulating in small-scale dairy farms in these states. To correlate farming practices with zoonotic risk, we combined results from a dairy farmer questionnaire with cow seroprevalence. However, low seroprevalence levels found in this study made the identification of risk factors difficult. Nevertheless, poor farming practices around hygiene and biosecurity on dairy farms have been highlighted. Implementing simple measures could mitigate environmental contamination, and therefore, reduce the risk of *Leptospira interrogans*, and other zoonoses transmission, at the animal-environment-human interface.

Keywords: dairy cows; leptospirosis; India; zoonoses; small-scale farms; seroprevalence

1. Introduction

Leptospirosis is an important re-emerging zoonosis across the world [1,2]. In India, leptospirosis is endemic in both human and animal populations [3,4], with the country listed as a global hot spot [5] for the disease. Non-specific clinical symptoms of leptospirosis in both humans [6] and animal populations, specifically livestock, is problematic [7], contributing to underreporting and misdiagnosing of this zoonotic disease in India [8,9]. In recent years, there is increasing interest in strengthening human leptospirosis surveillance systems [10]; however, this focuses on human cases only and neglects the numerous animals who act as carriers, as well as environments contaminated from infected animals [11,12]. Such human-based surveillance approaches are problematic in endemic states of India where cattle have been identified as important hosts for several *Leptospira* serovars [8,9,13].

Pathogenic serovars of *Leptospira interrogans* are the most common found in cattle [7,13], of note is *Leptospira interrogans* serovar *Hardjo*, which causes important reproductive losses and economic impacts at the farm level [1,13,14]. In India, vaccination against leptospirosis in cattle is currently not practiced [7]. Previous bovine studies have adopted a seroepidemiological approach, describing very different *Leptospira* spp. infection patterns across Indian states [1,15–17]. Factors such as geographical locations [18], farm reproductive histories [12], and diagnostic methods used [19,20] all contribute to a varied understanding of bovine leptospirosis seroepidemiology. High antibody titres for leptospirosis in human patients have been found in the states of Bihar and Assam in the North-Eastern province of India [21]. These states suffer from heavy rainfall and severe floods, believed to cause increased regional transmission risks for humans [2,10]. Despite vast cattle populations in these states [22] and the increased occupational risk of *Leptospira* spp. infection in dairy farmers, exposed through working barefoot, manually milking, and lacking protective clothing [7,14,23], the role of dairy farms in maintaining and transmitting this zoonotic pathogen, has received little attention thus far.

Studies in a developing world context, which focus solely on serology, fail to give sufficient insight into sustainable disease control options [24]; to understand leptospirosis epidemiology in cattle and indeed the zoonotic risk to humans, observing more than bovine serological prevalence is needed [25]. To this end, a farmer questionnaire was developed in this study to investigate farming practices on small-scale dairy farms in Assam and Bihar. Assessing knowledge, attitudes, and practices (KAPs) of livestock keepers has proven helpful in the design of control strategies and health education campaigns for other diseases [26], with research showing how good farmer knowledge, attitudes, and practices relating to leptospirosis improved initiation of relevant control measures [27]. Therefore, understanding farmer behaviour on farms will allow for sustainable intervention measures to be implemented.

The current lack of data on zoonotic pathogens at the farm level is hindering farmers, agricultural organisations, veterinary and medical services in evaluating the need for implementing disease control procedures in North Eastern India [28]. The objective of this cross-sectional study was to not only generate another estimate of bovine *Leptospira interrogans* serovar *Hardjo* seroprevalence but to contextualise seroprevalence levels within these two states through evaluating dairy farmers practices and behaviour and create an integrated overview of host-pathogen interactions, to determine potential pathways to mitigate leptospirosis transmission.

2. Materials and Methods

2.1. Sampling Design

A cross-sectional study was conducted in the two North-East Indian states Assam and Bihar from 2015–2016 using a multistage sampling technique for household sample selection in both states. The selection has been described in detail before [28], but in short three districts, each from the 33 districts in Assam and 38 districts in Bihar, were selected, guided by consultations with the Animal Husbandry and Veterinary Department officials of each state given the district's potential for dairy development. Availability of primary laboratory support, as well as safety of the study team, were also considered during the selection of the districts. Accordingly, Kamrup, Golghat, and Baska districts were selected from Assam and Patna, Nalanda, and Vaishali districts were selected from Bihar. In the second stage, two community development blocks (CDBs) from each district (one rural and one urban) were selected. In Patna district, one peri-urban CDB was also included. Thus, a total of 6 CDBs in Assam and 7 CDBs in Bihar were selected. From each CDB, 4 villages were selected randomly from villages with sizeable dairy cattle and buffalo populations, and from each selected village, 10 households having large ruminants (cattle and buffalo) were selected randomly from a list.

Assuming household level estimate of the prevalence to be 15% for infection, at 95% level of confidence and 5% precision in the estimates, the sample size was calculated to be 196 and to account for a small design effect because of clustering, the aim was to target 240 households in each state [29].

2.2. Ethical Approval

The study was approved by Institutional Research Ethics Committee (IREC) at International Livestock Research Institute (ILRI) ILRI-IREC 2015-12 and IREC 2017-01, IACUC 2017-05, as well as the Institutional Animal Ethics Committee, Indian Council of Agricultural Research (ICAR) and the CGIAR Research Programme on Agriculture for Nutrition and Health. All the farm owners were informed about the study and consented to participate and for data to be published.

2.3. Data Collection

During fieldwork, 534 dairy farmers were interviewed in the local language using a pre-tested questionnaire with closed questions to collect data on demographics, as well as on farming systems, milking, and husbandry practices. The questionnaire was piloted in the area before the start of the survey, and the interviewing personnel were trained to use a common methodology. From each household, up to three adult lactating cows were selected for sampling as described previously [28], samples were transported to ICAR-NIVEDI, Bangalore, and stored at −20 °C until tested. Animal data and data on clinical signs were collected to correlate with seroprevalence.

2.4. Serological Sampling

The serum samples from 680 cows and 22 buffaloes were tested for *Leptospira* serovar *Hardjo* in serum by Priocheck *L. Hardjo* Ab indirect ELISA kit, Prionics AG, Switzerland [30]. The use of an ELISA antibody test showed a 90% correlation to MAT in one study and was deemed a good alternative for diagnosing leptospirosis [31].

Samples were run in singles following the manufacturer's instructions, results were interpreted based on percent positivity (PP). PP = Corrected optical density (OD) of test serum/Corrected optical density (OD) of reference test serum × 100:

PP = <20%: Negative for *L. Hardjo* specific antibodies

PP = 20–45: Inconclusive results (antibodies may be present)

PP = >45%: Positive for *L. Hardjo* specific antibodies

A random set of 19 samples were run blindly in duplicates with no conflicting results, to control for repeatability.

2.5. Statistical Analysis

Data were entered into Microsoft Excel and analysed using 'R' statistical software [31]. All 22 buffalo samples were seronegative. Given the scarcity of buffalo numbers, combined with a serovar-specific species study approach, these 22 samples were excluded from the statistical analyses. Therefore 680 cows from 531 farms were used in the final analysis. Initial univariable analyses were conducted using Chi2 or Fisher's exact testing to identify each potential risk variable for *Leptospira* serovar *Hardjo* seropositivity. For the univariable analyses, it was decided to treat the inconclusive values as seronegative samples, to not risk a bias away from null. Given the low level of seroprevalence and the low number of risk factors with a $p < 0.1$ in the univariable analyses (Table 1), a multivariable model was not conducted.

Table 1. Univariable analysis for risk factors associated with *L.* serovar *Hardjo* farm seroprevalence at $p < 0.1$.

Risk Factors	Risk Variables	Number of Positive Farms and Seroprevalence (%)	95% Confidence Interval	*p*-Value *
State	Bihar	13/289 (4.5%)	2.4–7.6	$p = 0.04$
	Assam	3/243 (1.2%)	0.26–3.6	
District	Kamrup	2/81 (2.5%)	0.30–8.6	$p = 0.07$
	Golghat	1/81 (1.2%)	0.03–6.7	
	Baska	0/80 (0%)	0–4.5%	

Table 1. *Cont.*

Risk Factors	Risk Variables	Number of Positive Farms and Seroprevalence (%)	95% Confidence Interval	p-Value *
	Patna	6/121 (5.0%)	1.8–10.4	
	Nalanda	6/86 (7.0%)	2.6–14.6	
	Vaishali	1/82 (1.2%)	0.03–6.6	
Floor type	Concrete Earthen Other	10/250 (4.0%) 2/203 (0.99%) 4/77 (5.2%)	1.9–7.2 0.12–3.5 1.4–12.8	$p = 0.06$
Farms where cows can have contact with dogs	Yes No	1/147 (0.68%) 15/384 (3.9%)	0.02–3.7 2.2–6.4	$p = 0.08$

* Fisher's exact test.

3. Results

In total 531 dairy farms were included in this study, 243 in the state of Assam and 288 in the state of Bihar, and 3 farms with buffalos only were excluded. We sampled 680 dairy cows, 337 cows in Bihar, and 343 in Assam. Figure 1 below shows study districts in Bihar and Assam. Of the 531 dairy farmer respondents to the questionnaire, 38 were female (7%, CI 5–10) and 493 were male (93%, CI 90–95%). The mean number of people living in a farming household was seven (SD 3.38). Of the farmers interviewed, 49% were between 41–60 years of age, 29% were between 21–40 years, 19% were over 60 years and 3% were under 20 years of age. Farmers' education level varied with 43% attending school for 5–10 years, 23% attending for 11–12 years, 13% had graduate-level studies, 11% had attending school for 5 years or less and 10% had never attended school.

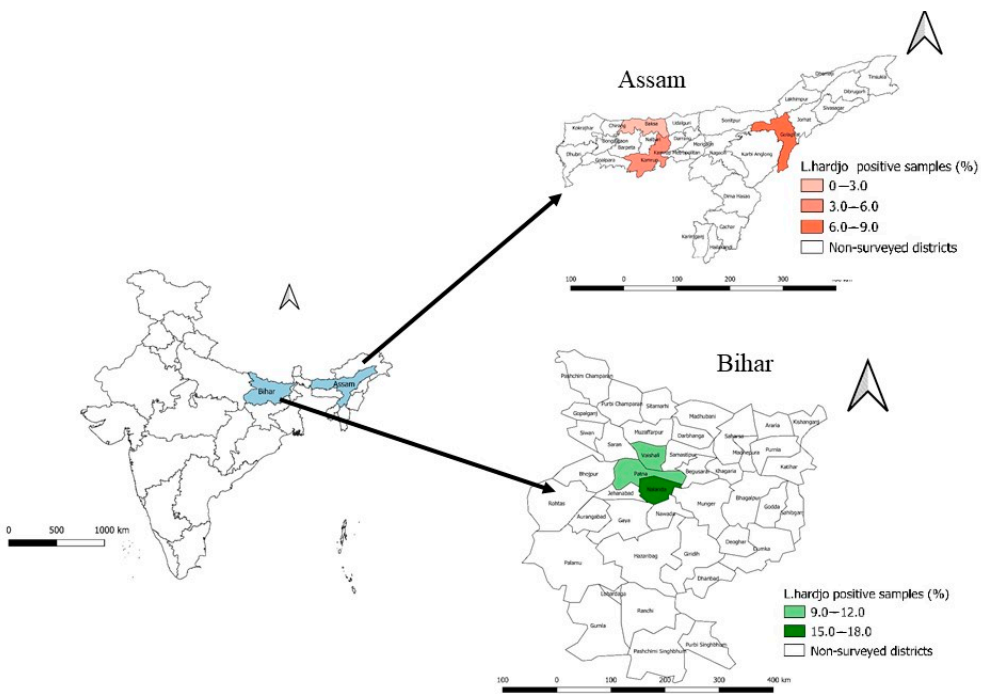

Figure 1. Seroprevalence for *L.* serovar *Hardjo* on dairy farms in the states of Bihar and Assam, India.

Of the 680 blood samples tested, results from the *L. Hardjo* Ab. indirect ELISA showed 16 seropositive results, 629 seronegative results, and 35 inconclusive results. Of the 16 seropositive results, 13 were from cows in Bihar (3.9% seroprevalence, 95% CI 2.1–3.5) and 3 from cows in Assam (0.9% seroprevalence, 95% CI 2.1–6.5). Of the 629 seronegative results, 300 were from cows in Bihar and 329 from cows in Assam. Of the 35 inconclusive results, 24 were from cows in Bihar and 11 from cows in Assam.

If the inconclusive results would be considered positive, the seroprevalence in Bihar would be 11.3% (38/337, 95% CI 8.1–15.1%), and in Assam 3.8% (13–343, 95% CI 2.0–6.4%). Seropositive results for 16 cows from 16 different dairy farms were found. Significantly ($p < 0.03$) more farms had positive cows in the state of Bihar (13 cows, 3.9%, 95% CI 2.3–6.5%; 13 farms, 4.5 %, 95% CI 2.6–7.5%) than Assam (3 cows, 0.87%, 95% CI 0.3–2.5%; 3 farms, 1.2%, 95% CI 0.42–3.6%). At the district level, Nalanda district in the state of Bihar had higher seroprevalence on farms (6 cows, 7.2%, 95% CI 3.3–15% and 6 farms, 7%, 95%CI (3.2–14%) than other districts (Table 1). Univariable analysis was carried out to identify farm variables associated with farm seroprevalence, state, district, floor type, and farms where cows were in contact with dogs were found to be associated with seroprevalence risk (Table 1).

3.1. Risk Factors Associated with Farm Seroprevalence

Farmer gender, age, and education level were not associated as risk factors for farm seroprevalence ($p > 0.5$, $p > 0.7$, $p > 0.3$, respectively). Farm size was categorised as small (<4 milking or dry cows), medium (4–10 milking or dry cows), or large (>10 milking or dry cows), where 77% (412) of farms fell into the small farm size category, 17% (88) were medium-sized and 6% (31) were large-sized farms. Farm size was not identified as a risk factor for seroprevalence ($p > 0.2$). Floor types in cowsheds were categorised into concrete, earthen, or other; 47%(250) of farms reported to have a concrete floor, 38% (203) had an earthen floor 1% (4) had floors of other materials, and 14% (74) farms had no report on floor type. Farms, where dairy cows are kept on concrete floors, had ($p = 0.08$) higher seroprevalence than farms with earthen or other floor types.

Rearing systems on the farms were described as fully stalled (59%, 315 farms) or partly stalled with part-time grazing (41%, 216 farms); neither system was identified as a risk factor for seroprevalence ($p = 1$). Among the 26% (140) of farms who introduced new cattle into their herds over the last 12 months, small farms significantly ($p < 0.001$) introduced more cattle than medium and large size farms but the introduction of new animals was not associated as a risk factor ($p > 0.2$). Out of the 140 farms that had introduced new animals, 37% (52) reported having done so from an unknown source. Artificial insemination (AI), as opposed to natural mating with a shared bull, was the predominant method used for breeding cows on 74% of farms, it was not associated with infection risk ($p > 0.3$). Significantly more ($p < 0.01$) small-sized farms vaccinate their herds against foot and mouth disease (FMD) than medium and large-sized farms, vaccination was not associated with farm seroprevalence. Regarding milking practices, all 100% (531) of farms use the hand milking method with a mean of 1.4 (SD 0.86) persons in charge of milking. Moreover, 9% (50) of farmers reported never cleaning the udder before milking, 91% (481) reported doing it infrequently, no farms reported doing it as routine practice. Udder hygiene was not associated as a risk factor for farm seroprevalence ($p > 0.4$).

After a normal calving or abortion, 45% (238) of farmers report to throw away the placenta, with 55% (288) disposing of it by burying it. While disposal of cow placenta was not found to be a risk factor for farm seroprevalence ($p > 0.15$), correct disposal should be more actively encouraged.

Only one of the seroprevalent dairy farms allowed mixing between cows and dogs and this was not identified as a risk factor as seen in Table 1. Contact between cows and other species; sheep, goats, pigs, cats or poultry, was also not associated with seroprevalence ($p > 0.45$, $p > 0.3$, $p > 1$, $p > 0.3$, $p > 0.4$, $p = 1$). Moreover, 38% (202) of farms reported possible contact between their cows and wild mammals such as rats and boars, this was not associated with increased seroprevalence risk ($p > 0.4$).

3.2. Risk Factors Associated with Cow Seropositivity

The mean age of seronegative cows was 5.48 (SD 2.3) years, and 5.94 years (SD 2.2) for seropositive cows, with a mean number of lactations of 2.5 (SD 1.2) for both seropositive and seronegative cows. Age above 5 years was significantly (p = 0.01) associated with increased seropositivity. Of the 16 seropositive cows, one cow was identified as being a local breed and the remaining 15 were crossbreds. The mean body condition score (BCS), which was scored from 0–5, for seropositive cows was 4 (SD 0.78), neither breed (p > 0.1) nor BCS (p = 1) were identified as risk factors for seropositivity. Clinical histories on the 680 cows showed that 6% (40) were reported to have aborted in the last 12 months, 16% (108) were reported to have repeat breeding, 4% (28) had retained placenta after calving and only one cow (0.1%) was reported to have had a stillbirth. Of the 680 cows, 69 were reported to have signs of mastitis during the last 12 months, for 374 cows an unknown history of mastitis was reported. For the 16 seropositive cows, their clinical histories for the previous 12 months showed that one had aborted, three had retained cleanings, and four had suffered mastitis. No association was found between seropositive cows and the presentation of these clinical signs (p = 1 for abortion, retained cleaning, mastitis). Retained placenta or stillbirths were not reported in any of the seropositive cows.

Of the 16 cows seropositive for *L.* serovar *Hardjo*, eight were found to have seropositive results for *Coxiella burnetii*, another zoonotic bacterial pathogen. When we compared these two categorical variables, we found that having an *L.* serovar *Hardjo* positive result was significantly associated with having a *Coxiella burnetii* seropositive result (p < 0.001).

For the eight cows that had both *L.* serovar *Hardjo* and *C. burnetii* seropositive results, we examined if they had clinical histories of abortion, repeat breeding or mastitis, to see if cows with both pathogens were more at risk of presenting with clinical signs but there was no significant association between clinical signs with comorbidity (p = 1). Of these eight seropositive cows for both *L.* serovar *Hardjo* and *C. burnetii*, one cow was reported to have aborted, three were reported to have had repeat breeding and two had reported signs of mastitis. Table 2 highlights the variables of state, district, age of cows, and presence of other pathogens, as these were found to be risk factors associated with *L.* serovar *Hardjo* cow seropositivity.

Table 2. Univariable analysis for risk factors associated with *L.* serovar *Hardjo* cow seropositivity.

Risk Factors	Risk Variables	Number of Positive Cows/Total Tested (Seropositivity %)	95% Confidence Interval	*p*-Value
State	Bihar	13/337 (3.9%)	2.1–6.5	p = 0.01 [a]
	Assam	3/343 (0.87%)	0.2–2.5	
Districts in Assam	Kamrup (capital)	2/163 (1.2%)	0.15–4.4	p = 0.024 [b]
	Golghat	1/90 (1.1%)	0.03–6.0	
	Baska	0/90 (0%)	0–4.0	
Districts in Bihar	Patna (capital)	6/158 (3.8%)	1.4–8.1	
	Nalanda	6/84 (7.15%)	2.7–14.9	
	Vaishali	1/95 (1.1%)	0.03–5.7	
Age levels	Cows >5 years	15/404 (3.7%)	2.1–6.0	p = 0.005 [a]
	Cows <5 years	1/274 (0.36%)	0.009–2.0	
Serological status for *Coxiella burnetii*	Negative	8/571 (1.4%)	0.61–2.7	p < 0.001 [a]
	Positive	8/92 (8.7%)	3.8–16.4	

[a] Chi-2 test, [b] Exact Fisher test.

4. Discussion

The bovine seroepidemiological scenario described in our study corresponds to that of other studies in India and beyond [4,7,25] where geographical location is an influencing factor in bovine leptospirosis prevalence [7,16,20]. Our study found varying serological prevalence between states and within districts. We found higher seroprevalence on small-scale dairy farms in Bihar 4.5% (95% CI 2.6–7.5%) than Assam 1.2% (95% CI 0.42–3.6%), and higher seroprevalence within the Nalanda district of Bihar (7%, 95% CI 3.2–14%). Previously, only large-scale organised dairy farms in other states had been investigated for leptospirosis prevalence, describing seroprevalences in the different states between 3.7 and 30.4% [7]. With dairy cattle acting as important reservoirs of *Leptospira interrogans* serovar *Hardjo* [12,17,32,33] and Indian dairy farmers being highly exposed to *Leptospira* spp. [3,9]; this cross-sectional study allows for a timely, first estimate of apparent prevalence among small-scale dairy farms in Assam and Bihar. The prevalence was towards the lower end of the reported range suggesting small farm size may be risk-mitigating.

Microscopic agglutination test (MAT) is regarded as the gold standard reference serological test for bovine leptospirosis [34] but maintaining large numbers of serovars of *L. interrogans* for the MAT method is a logistical and financial challenge [7]. The use of the *Leptospira* serovar *Hardjo* indirect ELISA kit in this study avoided additional expense and time in comparison to the MAT method [35], acting as a quick and inexpensive screening tool [7,16], thereby providing an insight into how larger-scale, future herd screening programmes could be achieved. The interpretation of a single indirect ELISA result, as done in this study, must be done with caution; low antibody titres do not exclude a diagnosis of leptospirosis, as titres are often low in acute disease and maintenance hosts [36], and the test is focused of only one of many serovars. A second round of sampling should be incorporated into future study designs to offset this diagnostic limitation. This study only included ELISA results as positive if they were above the threshold, and all inconclusive results were considered negative, as it was not possible to re-test the animals. This likely gave a lower prevalence than in reality, and it could have biased the analyses of associations, but likely more towards null.

Farmers rely heavily on livestock production in the states of Bihar and Assam [37] bringing to the fore the importance of understanding human-animal contact patterns in these areas if zoonotic disease risks are to be reduced [38]. Serological surveillance has a role; however, understanding farming practices is key for developing long-term, effective control strategies [27]. The proximity between cattle and their human handlers, especially on dairy farms [39] makes dairy cattle a species of particular interest when investigating leptospirosis transmission pathways [40]. Understanding transmission dynamics and the cultural characteristics of a community faced with the problem of leptospirosis [41] will mitigate transmission risks at the animal-environmental interface humans [9]. Using a farmer knowledge, attitudes, and practices questionnaire, an insight into animal-human contact patterns was achieved in this study.

The results from our univariable analysis identified only four risk factors for seroprevalence; state, district, floor type, and contact between cows and dogs. Low seroprevalence found overall, especially in Assam, contributed to few risk factors being identified. Nevertheless, this does not undermine the important insights we gained into farming practices on small-scale dairy farms. Poor milking hygiene and substandard biosecurity practices were apparent from the farmer KAP responses, an important finding given how farming practices are intrinsically linked to animal and human health [42,43].

Our study found that 45% of the farmers interviewed did not dispose of the placenta correctly (i.e., bury it) after abortion or calving. *Coxiella burnetii*, *Chlamydia abortus*, *Brucella* spp. and *Leptospira* spp. are just some bacterial zoonotic agents capable of contaminating the environment through placentas and postpartum fluid [44–46], thereby contributing to the spread of infection at the human-cattle nexus [46]. Moreover, 28% of dairy cows in this study have contact with dogs and 19% have contact with cats, with dogs and cats recognised as potential mechanical vectors of infected bovine material [47], and such mixing of species could increase environmental contamination.

The presence of a second bacterial zoonotic pathogen, *Coxiella burnetii*, was found to be associated with increased *Leptospira interrogans* serovar *Hardjo* seropositivity in cows in this study. No other study in these states of India has investigated this co-morbidity in cattle to the authors' knowledge. The presence of a second zoonotic agent on these dairy farms highlights potential multiple disease transmission pathways. Poor knowledge of the disease, combined with high-risk behaviours, strengthens the logic for including health education as part of zoonoses control programmes [27,46].

All 531 dairy farmers in this study hand milk, and no farmer reported always, routinely cleaning the udder before milking. Improved udder hygiene in small scale-dairy farms, in combination with other measures, lowers the prevalence of contagious pathogens on the udder and in the milk [48]. Other bovine zoonotic pathogens such as *Listeria* spp., *Salmonella* spp., *Staphylococcus aureus,* and *Streptococcus* spp. and livestock-associated methicillin-resistant *S. aureus* are transmitted through direct cow-human contact [46] underpinning the link between udder health and farmer health. Udder health appears to currently be poorly prioritized in these small-scale dairy farms, where 374 cows were reported to have an unknown history of mastitis, highlighting a possible problem in farmers' ability to recognise signs of mastitis. Improving knowledge, attitudes, and practices among small-scale dairy farmers could have a significant impact on the reduction of multiple zoonotic infections [27], not just leptospirosis, and improve farm productivity, reduced through poor milk yields and reproductive disorders caused by these bacterial pathogens [42,49].

In this study, none of the seropositive cows were found to be associated with clinical signs of disease, a complicating factor to leptospirosis control [11,25]. Lack of clinically affected cows hinders disease awareness and perception among farmers, and such a challenge must be a consideration for future disease control measures [12]. How will dairy farmers engage in control measures for a disease they do not visibly perceive to be present in their herd? Improved engagement between farmers and veterinary extension services needs to be achieved. Farmer-focused educational campaigns, highlighting the intrinsic economic link between poor production parameters; low milk yield, and long calving intervals, with poor disease control, should be incorporated into future study designs.

A limitation to the study design was the many missing values in farmer questionnaire responses; low responses to certain questions especially among farmers in the state of Bihar could indicate farmer questionnaire fatigue [50]. Future studies may yield more data if a multidisciplinary approach creating a more appropriate data collection model [51], such as the inclusion of social scientists, economists, and gender specialists in the farmer questionnaire design stage. Similarly, future studies should include a larger sample size, over more states to allow more conclusions about risk factors to be done.

In summary, leptospirosis seroprevalence in dairy cows was found to be low in this study making significant links between infection risk and farmer behaviour difficult to prove. Nevertheless, certain farming practices were identified which, if modified, could minimise the risk of other zoonotic infections [42] and mitigate drivers of disease at the farm level [52,53]. This study shows that at least two zoonotic diseases, leptospirosis, and coxiellosis, are co-circulating on small-scale dairy farms in the states of Assam and Bihar in North Eastern India. Surveillance systems to identify both human and animal *Leptospira* serovars are needed [21] combined with a deeper understanding of farming practices, as highlighted by this study, if transmission of leptospirosis and other zoonoses, such as coxiellosis, is to be mitigated at the human-animal-environmental interface.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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References

1. Prajapati, A.; Kushwaha, A.; Chayanika, D.; Subhashree, N.; Varsha, P.; Marcia, A.; Lahari, L.; Shankar, S.; Patel, N. A Review on Bovine Leptospirosis with Special Reference to Seroprevalence in India. *Int. J. Curr. Microbiol. Appl. Sci.* **2018**, *7*, 1813–1820. [CrossRef]
2. Chaudhry, R.; Premlatha, M.; Mohanty, S.; Dhawan, B.; Singh, K.K.; Dey, A. Emerging Leptospirosis, North India. *Emerg. Infect. Dis.* **2002**, *8*, 1526–1527. [CrossRef]
3. Natarajaseenivasan, K.; Vedhagiri, K.; Sivabalan, V.; Prabagaran, S.G.; Sukumar, S.; Artiushin, S.C.; Timoney, J.F. Seroprevalence of Leptospira borgpetersenii serovar javanica infection among dairy cattle, rats and humans in the Cauvery river valley of southern India. *Southeast Asian J. Trop. Med. Public Health* **2011**, *42*, 679–686. [PubMed]
4. Shivakumar, S. Leptospirosis—Current Scenario in India. In *Medicine Update*; API: Mumbai, India, 2008; Volume 106, pp. 799–809. [CrossRef]
5. Grace, D.; Mutua, F.; Ochungo, P.; Kruska, R.; Jones, K.; Brierley, L.; Lapar, M.L.; Said, M.; Herrero, M.; Pham-Duc, P.; et al. Mapping of poverty and likely zoonoses hotspots. *Zoonoses Proj.* **2012**, *4*, 1–119.
6. Jena, A.B.; Mohanty, K.C.; Devadasan, N. An outbreak of leptospirosis in Orissa, India: The importance of surveillance. *Trop. Med. Int. Health* **2004**, *9*, 1016–1021. [CrossRef]
7. Balamurugan, V.; Alamuri, A.; Veena, S.; Bharatkumar, K.; Patil, S.S.; Govindaraj, G. Investigation on the prevalence of leptosira serovars Hardjo in organized cattle dairy farms of India. *Indian J. Anim. Sci.* **2016**, *86*, 1145–1147.
8. Vijayachari, P.; Sugunan, A.P.; Shriram, A.N. Leptospirosis: An emerging global public health problem. *J. Biosci.* **2008**, *33*, 557–569. [CrossRef] [PubMed]
9. Sugunan, A.P.; Vijayachari, P.; Sharma, S.; Roy, S.; Manickam, P.; Natarajaseenivasan, K.; Gupte, M.D.; Sehgal, S.C. Risk factors associated with leptospirosis during an outbreak in Middle Andaman, India. *Indian J. Med. Res.* **2009**, *130*, 67–73.
10. Sethi, S.; Sharma, N.; Kakkar, N.; Taneja, J.; Chatterjee, S.S.; Banga, S.S.; Sharma, M. Increasing Trends of Leptospirosis in Northern India: A Clinico-Epidemiological Study. *PLoS Negl. Trop. Dis.* **2010**, *4*, e579. [CrossRef]
11. Martins, G.; Lilenbaum, W. Control of bovine leptospirosis: Aspects for consideration in a tropical environment. *Res. Vet. Sci.* **2017**, *112*, 156–160. [CrossRef]
12. Balamurugan, V.; Alamuri, A.; Bharathkumar, K.; Patil, S.S.; Govindaraj, G.N.; Nagalingam, M.; Krishnamoorthy, P.; Rahman, H.; Shome, B.R. Prevalence of Leptospira serogroup-specific antibodies in cattle associated with reproductive problems in endemic states of India. *Trop. Anim. Health Prod.* **2018**, *50*, 1131–1138. [CrossRef]
13. Pandian, S.J.; Ray, P.K.; Chandran, P.C.; Kumar, M. Seroprevalence of Brucella abortus and Leptospira hardjo in cattle. *Vet. World* **2015**, *8*, 217–220. [CrossRef] [PubMed]
14. Parvez, M.A.; Prodhan, M.A.; Rahman, M.; Faruque, M.R. Seroprevalence and associated risk factors of Leptospira interrogans serovar Hardjo in dairy cattle of Chittagong, Bangladesh. *Pak. Vet. J.* **2015**, *35*, 350–354.
15. Mandal, S.; Joardar, S.N.; Chakraborty, D.; Sardar, N. Seroepidemiological study of bovine leptospirosis in West Bengal. Indian, J. Comp. Microbiol. Immunol. *Infect. Dis.* **2008**, *29*, 42–44.
16. Patel, J.M.; Vihol, P.D.; Prasad, M.C.; Kalyani, I.H.; Raval, J.K.; Patel, K.M.; Sushma, R.A.; Thirumalesh, B.V. Seroepidemiological pattern of leptospirosis in bovine of South Gujarat, India. *Vet. World* **2014**, *7*, 999–1003. [CrossRef]
17. Patel, J.; Prasad, M.; Vihol, P.; Kalyani, I.; Prajapati, M.; Parmar, H.; Varia, R. Seroprevalence of Leptospira hardjo in Cattle of Gujarat, India. *Int. J. Curr. Microbiol. Appl. Sci.* **2017**, *6*, 1304–1310. [CrossRef]

18. Lau, C.L.; Smythe, L.D.; Craig, S.B.; Weinstein, P. Climate change, flooding, urbanisation and leptospirosis: Fuelling the fire? *Trans. R. Soc. Trop. Med. Hyg.* **2010**, *104*, 631–638. [CrossRef]
19. Rajan, B.; Kumar, S.; Pillai, R.M.; Antony, P.X.; Mukhopadhyay, H.K.; Balakrishnan, S.; Rajkumar, N.; Srinivas, M.V. Comparative study on serodiagnosis of bovine leptospirosis by microagglutination test (MAT) and indirect ELISA. *Int. J. Curr. Microbiol. App. Sci.* **2017**, *6*, 1551–1558.
20. Balamurugan, V.; Veena, S.; Thirumalesh, S.; Alamuri, A.; Sridevi, R.; Sengupta, P.; Govindaraj, G.; Nagalingam, G.; Hemadri, D.; Gajendragad, M.; et al. Distribution of serogroup specific antibodies against leptospirosis in livestock in Odisha. *Indian J. Anim. Sci.* **2017**, *87*, 546–551.
21. Chandy, S.; Kirubanandhan, L.; Hemavathy, P.; Khadeeja, A.M.; Kurian, S.J.; Venkataraman, K.; Mørch, K.; Mathai, D.; Manoharan, A. Serovar prevalence of Leptospira in semirural India and the development of an IgM-based indirect ELISA. *J. Infect. Dev. Ctries.* **2017**, *11*, 234–241. [CrossRef]
22. Robinson, T.P.; Wint, G.R.W.; Conchedda, G.; Van Boeckel, T.P.; Ercoli, V.; Palamara, E.; Cinardi, G.; D'Aietti, L.; Hay, S.I.; Gilbert, M.; et al. Mapping the Global Distribution of Livestock. *PLoS ONE* **2014**, *9*, e96084. [CrossRef] [PubMed]
23. Mj, N.; Sm, A. A Sero-epidemiological Survey of Brucellosis, Q-Fever and Leptospirosis in Livestock and Humans and Associated Risk Factors in Kajiado County-Kenya. *J. Trop. Dis.* **2016**, *4*, 215. [CrossRef]
24. Godfroid, J.; Al Dahouk, S.; Pappas, G.; Roth, F.; Matope, G.; Muma, J.; Skjerve, E. A "One Health" surveillance and control of brucellosis in developing countries: Moving away from improvisation. *Comp. Immunol. Microbiol. Infect. Dis.* **2013**, *36*, 241–248. [CrossRef] [PubMed]
25. Lilenbaum, W.; Martins, G. Leptospirosis in Cattle: A Challenging Scenario for the Understanding of the Epidemiology. *Transbound. Emerg. Dis.* **2014**, *61*, 63–68. [CrossRef] [PubMed]
26. Holt, H.R.; Eltholth, M.M.; Hegazy, Y.M.; El-Tras, W.F.; A Tayel, A.; Guitian, J. Brucella spp. infection in large ruminants in an endemic area of Egypt: Cross-sectional study investigating seroprevalence, risk factors and livestock owner's knowledge, attitudes and practices (KAPs). *BMC Public Health* **2011**, *11*, 341. [CrossRef] [PubMed]
27. Lindahl, E.; Sattorov, N.; Boqvist, S.; Magnusson, U. A Study of Knowledge, Attitudes and Practices Relating to Brucellosis among Small-Scale Dairy Farmers in an Urban and Peri-Urban Area of Tajikistan. *PLoS ONE* **2015**, *10*, e0117318. [CrossRef]
28. Shome, R.; Deka, R.P.; Milesh, L.; Sahay, S.; Grace, D.; Lindahl, J.F. Coxiella seroprevalence and risk factors in large ruminants in Bihar and Assam, India. *Acta Trop.* **2019**, *194*, 41–46. [CrossRef] [PubMed]
29. Naing, L.; Winn, T.; Rusli, B.N. Practical issues in calculating the sample size for prevalence studies. *Arch. Orofac. Sci.* **2006**, *1*, 9–14.
30. Prionics, A.G. ELISA for In Vitro Detection of Antibodies against Leptospira Interrogans Serovar Hardjo in Serum and Milk of Dairy Cattle. Switzerland. Available online: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0013802_7442080_UG_en.pdf (accessed on 6 July 2020).
31. Bercovich, Z.; Taaijke, R.; Bokhout, B. Evaluation of an ELISA for the diagnosis of experimentally induced and naturally occurring Leptospira hardjo infections in cattle. *Vet. Microbiol.* **1990**, *21*, 255–262. [CrossRef]
32. R Core Team. *R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing*; R Core Team: Vienna, Austria, 2021; Available online: http://www.R-project.org/ (accessed on 11 March 2021).
33. Mughini-Gras, L.; Bonfanti, L.; Natale, A.; Comin, A.; Ferronato, A.; La Greca, E.; Patregnani, T.; Lucchese, L.; Marangon, S. Application of an integrated outbreak management plan for the control of leptospirosis in dairy cattle herds. *Epidemiol. Infect.* **2013**, *142*, 1172–1181. [CrossRef]
34. Scolamacchia, F.; Handel, I.G.; Fèvre, E.M.; Morgan, K.L.; Tanya, V.N.; Bronsvoort, B.M.D.C. Serological Patterns of Brucellosis. Leptospirosis and Q Fever in Bos indicus Cattle in Cameroon. *PLoS ONE* **2010**, *5*, e8623. [CrossRef]
35. OIE Leptospirosis. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 2014. Available online: https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.01.12_LEPTO.pdf (accessed on 6 July 2020).
36. Wynwood, S.J.; Burns, M.A.; Graham, G.C.; Weier, S.L.; McKay, D.B.; Craig, S.B. Serological diagnosis of Leptospirosis in bovine serum samples using a microsphere immunoassay. *Vet. Rec. Open* **2016**, *3*, e000148. [CrossRef]
37. Lunn, K. Overview of Leptospirosis. 2018. Available online: https://www.msdvetmanual.com/generalized-conditions/leptospirosis/overview-of-leptospirosis (accessed on 6 July 2020).
38. Gupta, J.; Singh, K.; Bhatt, B.; Dey, A. A Diagnostic Study on Livestock Production System in Eastern Region of India. IDEAS Working Paper Series from RePEc. 2014. Available online: https://mpra.ub.uni-muenchen.de/59517/1/MPRA_paper_59517.pdf (accessed on 8 July 2020).
39. Klous, G.; Huss, A.; Heederik, D.J.; Coutinho, R.A. Human–livestock contacts and their relationship to transmission of zoonotic pathogens, a systematic review of literature. *One Health* **2016**, *2*, 65–76. [CrossRef]
40. Hemsworth, P.; Coleman, G. Human-Livestock Interactions. In *The Stockperson and the Productivity and Welfare of Intensively Farmed Animals*, 2nd ed.; CABI: Cambridge, MA, USA; Wallingford, UK, 2011.
41. Mwachui, M.A.; Crump, L.; A Hartskeerl, R.; Zinsstag, J.; Hattendorf, J. Environmental and Behavioural Determinants of Leptospirosis Transmission: A Systematic Review. *PLoS Negl. Trop. Dis.* **2015**, *9*, e0003843. [CrossRef]
42. Bharti, A.R.; E Nally, J.; Ricaldi, J.N.; A Matthias, M.; Diaz, M.M.; A Lovett, M.; Levett, P.N.; Gilman, R.H.; Willig, M.R.; Gotuzzo, E.; et al. Leptospirosis: A zoonotic disease of global importance. *Lancet Infect. Dis.* **2003**, *3*, 757–771. [CrossRef]

43. Jones, B.A.; Grace, D.; Kock, R.; Alonso, S.; Rushton, J.; Said, M.Y.; McKeever, D.; Mutua, F.; Young, J.; McDermott, J.; et al. Zoonosis emergence linked to agricultural intensification and environmental change. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 8399–8404. [CrossRef] [PubMed]
44. McKenzie, J.S.; Dahal, R.; Kakkar, M.; Debnath, N.; Rahman, M.; Dorjee, S.; Naeem, K.; Wijayathilaka, T.; Sharma, B.K.; Maidanwal, N.; et al. One Health research and training and government support for One Health in South Asia. *Infect. Ecol. Epidemiol.* **2016**, *6*, 33842. [CrossRef] [PubMed]
45. Smits, H.L.; Kadri, S.M. Brucellosis in India: A deceptive infectious disease. *Indian J. Med Res.* **2005**, *122*, 375–384. [PubMed]
46. Vidal, S.; Kegler, K.; Greub, G.; Aeby, S.; Borel, N.; Dagleish, M.P.; Posthaus, H.; Perreten, V.; Rodriguez-Campos, S. Neglected zoonotic agents in cattle abortion: Tackling the difficult to grow bacteria. *BMC Vet. Res.* **2017**, *13*, 373. [CrossRef]
47. McDaniel, C.J.; Cardwell, D.M.; Moeller, R.B.; Gray, G.C. Humans and Cattle: A Review of Bovine Zoonoses. *Vector-Borne Zoonotic Dis.* **2014**, *14*, 1–19. [CrossRef]
48. Wheelhouse, N.; Longbottom, D. Endemic and Emerging Chlamydial Infections of Animals and Their Zoonotic Implications. *Transbound. Emerg. Dis.* **2011**, *59*, 283–291. [CrossRef] [PubMed]
49. Omore, A.; McDermott, J.; Arimi, S.; Kyule, M. Impact of mastitis control measures on milk production and mastitis indicators in smallholder dairy farms in Kiambu district, Kenya. *Trop. Anim. Health Prod.* **1999**, *31*, 347–361. [CrossRef] [PubMed]
50. Vaidya, V.M.; Malik, S.; Bhilegaonkar, K.; Rathore, R.; Kaur, S.; Barbuddhe, S. Prevalence of Q fever in domestic animals with reproductive disorders. *Comp. Immunol. Microbiol. Infect. Dis.* **2010**, *33*, 307–321. [CrossRef] [PubMed]
51. Pennings, J.M.; Irwin, S.H.; Good, D.L. Surveying Farmers: A Case Study. *Rev. Agric. Econ.* **2002**, *24*, 266–277. [CrossRef]
52. Magrath, P. Methodologies for Studying Agricultural Markets in Developing Countries. Marketing, 1992. Available online: https://core.ac.uk/download/pdf/42389745.pdf (accessed on 6 August 2020).
53. IOM (Institute of Medicine); NRC (National Research Council). *Sustaining Global Surveillance and Response to Emerging Zoonotic Diseases*; The National Academies Press: Washington, UK, 2009.