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Reproductive disorders in relation to *Neospora caninum*, *Brucella* spp. and bovine viral diarrhoea virus serostatus in breeding and dairy farms of central and southern Ethiopia

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SUMMARY

Abortion and stillbirth are important reproductive disorders in the dairy industry and are often caused by infectious agents. This study investigated whether bovine viral diarrhoea virus (BVDV), *Brucella* spp., *and Neospora caninum* are associated with abortion and/or stillbirth in dairy cattle in Ethiopia. Dairy cattle from 99 farms were categorized as cases ($n=134$) or controls ($n=268$) according to reproductive data. Blood samples were screened for antibodies for these infectious agents. The overall proportion of cattle that were seropositive for BVDV, *Brucella* spp., and *N. caninum* was 11·7%, 3·2%, and 17·2%, respectively. Seropositivity for BVDV and *Brucella* spp. was similar for cases and controls, but significantly more cases were seropositive for *N. caninum* (29·8%) than controls (10·8%). This is the first report demonstrating *N. caninum* is common in dairy cattle in Ethiopia, and is probably a greater impediment to reproductive success in Ethiopian dairy farms than either BVDV or *Brucella* spp.

Key words: Animal pathogens, *Brucella*, parasites, veterinary epidemiology, virus infection.

INTRODUCTION

Reproductive health problems in intensively managed dairy cattle in Ethiopia have been reported to affect almost as high as 70% of cows [1]. In livestock production, abortion and stillbirth are two of the most notable clinical reproductive disorders globally [2]. Incidence of abortion and stillbirth in the dairy sector in Ethiopia ranges between 4·4% and 20·2%, where these disorders are recognized as being among the many challenges to this sector [1, 3]. According to available research evidence, nearly half of the cases of abortion or stillbirth worldwide are caused by infectious agents [2], including bovine viral diarrhoea virus (BVDV), *Brucella* spp., and *Neospora caninum* [4–6].

BVDV infection has long been associated with reproductive failure leading to abortion and/or stillbirth [5, 7]. Bovine brucellosis is one of the important diseases in livestock and humans in sub-Saharan Africa and is commonly associated with abortion [5]. In Ethiopia, it is one of the few infectious cattle diseases that have been relatively well surveyed [8–12], as it has traditionally been the pathogen most associated with reproductive failures. However, the low (<3%) prevalence of brucellosis reported in dairy and breeding farms seems insufficient to explain the

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magnitude of abortion and stillbirth recorded in this sector [1, 3, 11, 12]. Neosporosis is an infectious disease caused by the protozoan parasite *N. caninum*, for which canids are the definitive host. It is being increasingly reported as a leading cause of abortion in the dairy cattle industry [13–15]. In addition to abortion and stillbirth, fetuses may die *in utero*, become mummified, autolysed, or are born alive with clinical nervous signs [7, 16]. Moreover, there is growing evidence of synergism between *N. caninum* and BVDV in inducing abortion and stillbirth in dairy cattle [17, 18].

Published information from Africa emphasizes the importance of all these diseases in this continent [11, 19–21]. However, in Ethiopia, documentation on the status of BVDV is limited to a single published report [22], and there is no information available on *N. caninum* in Ethiopian cattle. Thus, while bovine brucellosis has been the subject of considerable research, the possibly more important infections, BVD and neosporosis, have been relatively neglected.

The aim of this study was to investigate the serostatus of BVDV, *Brucella* spp., and *N. caninum* in central and southern Ethiopia and to estimate the magnitude of the risk of abortion and/or stillbirth associated with seropositivity to these infections. In addition, we describe some individual animal-level covariates in relation to exposure status for these infectious agents.

MATERIAL AND METHODS

Study area

This study was conducted in commercial dairy and state-owned breeding farms in 10 districts of central and southern Ethiopia. The farms were located between 6° 45′ to 9° 04′ North and 37° 44′ to 39° 16′ East. The altitude of the study area ranges from 1600 metres above sea-level (masl) to 2500 masl (Fig. 1). Most of the dairy farms are established in and around cities or towns. The commercial dairy and breeding farms serve as sources of breeding stock to small-scale urban and peri-urban dairies that have been established in adjacent districts.

Target population and study sample

The target population consisted of dairy and breeding farms composed of Friesians, Jerseys and their crossbreeds, located in and around major urban settings in the study area. The study samples were from farms with a history of abortion and/or stillbirth that had been reported to the district veterinary department. Only farms with $\geqslant 5$ animals were eligible for participation. In order to incorporate more farms (clusters), the number of cases per farm was limited to a maximum of two and the number of controls per farm to four in urban and peri-urban and commercial farms, while a maximum of six cases and 12 controls were selected from breeding farms. In this design, individual matching was intentionally avoided due to the anticipated problem of availability of controls in the same farm as most of the herds consisted of few animals.

Study design and sample size determination

The study was designed as a case-control study, in which cows or heifers which had experienced abortion, stillbirth or both were defined as cases. Controls were from the same herd but had no record of abortion and/or stillbirth. In order to minimize inclusion of control animals with antibodies from maternal passive transfer, only animals aged >6 months were eligible as controls. For the purpose of this study abortion was defined as loss of the fetus between 42 and 260 days of gestation, and stillbirth was defined as a calf that was born dead between 260 days and full-term, or died within 24 h following birth. The necessary minimum sample size was calculated using AusVet [23] based on a case-control study design with a predetermined odds ratio (OR) of 3, an expected prevalence of exposure in control groups of 10%, a desired level of confidence 95%, precision 5%, and a power of 80%, thus leading to a sample size of 97 cases. With two controls selected per case, the number of controls should have been 194. However, due to an expected cluster (herd) effect, and increasing the power by using two controls per case, the sample size was increased by $>35\%$. Thus, a minimum of 134 cases and 268 controls were selected to be enrolled in this study. In selecting the actual sample, the sampling frame of herds with abortion or stillbirth was prepared in collaboration with 10 relevant city or district veterinary departments. In each city, depending on the number of farms in the sampling frame prepared, 10–50% of herds were chosen at random. In each herd or farm where one or two cases were found, these were selected and controls were chosen at random. However, in farms with many cases, both case and control group animals were selected randomly after registration. Following final selection, cattle from 99 farms were included in the study.

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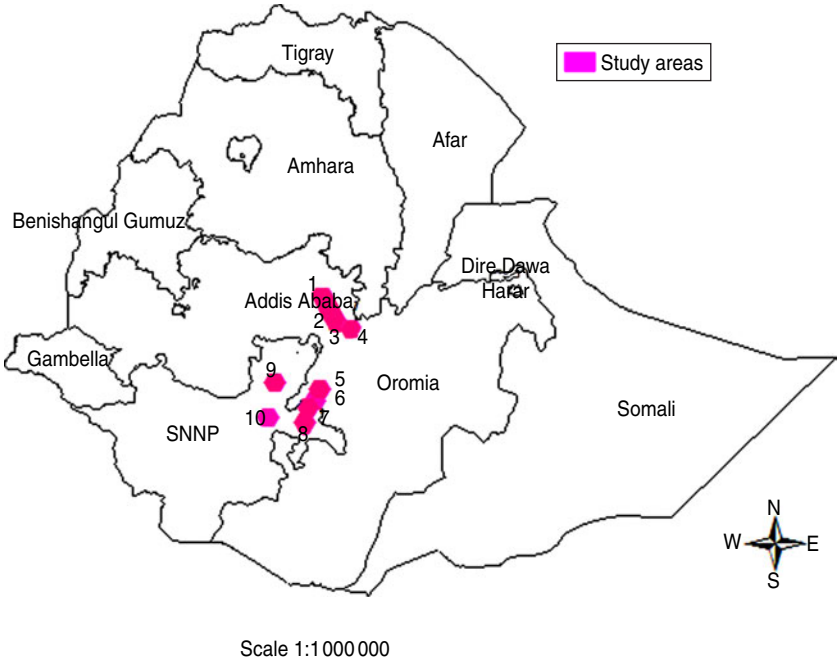

Fig. 1 [*colour online*]. Map of Ethiopia showing the location of the study farms. Central Ethiopia: 1, Holeta; 2, Addis Ababa; 3, Debrezeit; 4, Nazreth. Southern Ethiopia: 5, Arsinegele; 6, Shashamane; 7, Hawassa; 8, Yergalem; 9, Hossena; 10, Wollitasodo.

Blood sampling and serological screening

Cattle were bled from the jugular vein using sterile needles and Vacutainer tubes. The tubes, containing between 7 and 10 ml blood, were allowed to stand overnight at room temperature before being centrifuged at 1000 ***g*** for 15 min. The farm and animal identification codes were transferred to the cryovials to which the serum was decanted, and the serum samples were transported on ice to National Veterinary Institute, Debrezeit, and kept at −20 °C until screened. The presence of antibodies to *N. caninum* was determined using the IDEXX Neospora X2 Ab test kit (IDEXX, USA). A serum with absorbance value (S/P) with a cut-off level of ⩾0·50 was considered to be *Neospora* positive. For *Brucella*, antibody screening was conducted using the IDEXX Brucellosis Serum X2 Ab test kit and interpretation was based on S/P% where <110% was considered negative, 110–120% doubtful, and >120% positive. The test was repeated for doubtful results. BVDV exposure status was determined based on a competitive ELISA using the PrioCHECK BVDV-Ab test. Those samples whose percent inhibition (PI) was <50 were considered negative, while those with PI ⩾50% were considered positive. As there was no history of vaccination for the relevant agents at any of the study farms, seropositivity was assumed to be due to natural exposure. The test protocol and interpretation of all ELISA tests were performed according to the manufacturer's instructions (IDEXX).

Epidemiological information and data analysis

Individual cow biodata and reproductive performance records were collected for both cases and controls. If no written record was available, owners were interviewed using a semi-structured questionnaire, in which both herd and individual animal data were included. Data gathered included the animal's age, breed, origin, and history of maternal reproductive disorders. For reproductive performance parameters, the data obtained were age at first service, number of services per pregnancy, age at first pregnancy, parity, calving interval, days open, type and frequency of reproductive disorder encountered (abortion, stillbirth, uterine infection, retained fetal membranes, repeat breeding, birth of weak or defective calf, dystocia, and uterine or vaginal prolapse).

Animals were categorized according to the 'ever' *vs.* 'never' basis. Biodata categorization included breed (Friesian, Friesian cross, Jersey), and age (calf, young, heifer, adult). Information on reproductive performance indicators (age at first service, age at first pregnancy, calving to pregnancy interval) could not be used due to the lack of reliable records at most

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farms. However, parity number and associated clinical disorder both for the dam and calf, like abortion, stillbirth, retained fetal membranes, dystocia, prolonged uterine discharge, and birth of defective and weak calf were also categorized as either present or absent. 'Calving interval' was estimated from owners' information regarding the last consecutive calving as 'expected' or 'prolonged'. In this study, *expected* refers to calving every 12–18 months while *prolonged* refers to >18 months. Number of services per pregnancy was also estimated by owners. The presence or absence of a history of maternal reproductive disorders, if known, was categorized binomially. Repeat breeding was also assessed based on the owner's general observations and number of services required to establish pregnancy.

A database was established in Microsoft Excel®, for preliminary descriptive analyses, next the data were transferred to Stata SE/11 for Windows [24] for further statistical analysis. Associations between reproductive health problems and agent exposure (measured as serological status) were assessed using univariable logistic regression analysis, including covariates as age, parity, breed, origin and maternal reproductive disorder history. Considering each factor's biological plausibility in addition to their statistical relevance, a final multivariable logistic regression was built [25], using the backward elimination procedure to include variables in the model (inclusion criteria $P \leqslant 0{\cdot}05$). In the analysis, a covariate was considered to be a confounder and included in the model if its inclusion altered the OR of the estimated risk by $\geqslant 30\%$. The model was established using the matching stratum, with herd as the random effect. Finally, how well the model fitted with the observed data was evaluated using the Hosmer–Lemeshow test by the default approach, categorizing the data into 10 groups. Subsequently, the predictive ability of the model was validated using the receiver-operating characteristic (ROC) curve [25].

RESULTS

The final study sample included 402 breeding cattle, of which 134 were categorized as cases due to a history of abortion and/or stillbirth and 268 were categorized as controls, with no history of these disorders. Of the 268 controls, 11·6% ($n=31$) were aged between 6 and 16 months, but the remainder of the controls were older. Most of the cattle were Friesian ($n=344$), but some were Friesian crosses ($n=27$) or Jersey ($n=31$).

The percentages of cattle that were seropositive for BVDV, *Brucella* spp., and *N. caninum*, or combinations of these, are described in Table 1. *Neospora*-positive animals occurred more frequently ($P<0{\cdot}001$) in cases (29·8%) than controls (10·8%), while no differences were seen between cases and controls for *Brucella* spp. and BVDV reactors. Herd-level data are described in Figure 2, and demonstrate a similar pattern to those at the individual level. No geographical trends were observed.

Associations between the various different reproductive disorders reported (other than stillbirth and abortion) and the serological status for the three infections are described in Table 2. Some reproductive disorders (delivery of congenitally defective calf, birth of weak calf, dystocia, uterine or vaginal prolapses) were not associated with any of the three pathogens, but a range of disorders (prolonged calving interval, abortion, retention of fetal membranes, uterine infection) were associated with *Neospora* positivity. Figure 3 provides a web illustration of the frequency profile of important disorders for the three infection groups. Notably, the profile is very similar for *N. caninum* and BVDV.

The associations between individual animal covariates, the three infections and record of abortion and/or stillbirth are shown in Table 3. No difference was seen between cases and controls in terms of age, breed, and parity. Pluriparous animals were significantly associated with a record of abortion and/or stillbirth, and exposure to *N. caninum* (seropositive) was associated with history of maternal reproductive disorder and purchased animals. Associations between age and maternal history of abortion and/or stillbirth and *Neospora* seropositivity were also examined in the multivariable logistic regression model (Table 4). Further analysis focused on presenting the risk of various categories of reproductive disorders from *N. caninum* infection. The final model (Table 5), based upon results presented in Tables 1 and 2 and adjusted for potential covariates, shows associations between *Neospora* seropositivity and the different reproductive disorders.

DISCUSSION

This study demonstrates that cattle on the farms involved in this study in central and southern Ethiopia

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Table 1. *The serological status of BVDV,* Brucella *spp. and* Neospora caninum *in cases* (*history of stillbirth and/or abortion*) *and controls*

Infectious agents	Percentage seropositive				
	All animals ($n=402$)	Cases ($n=134$)	Controls ($n=268$)	OR*	*P* value*
BVDV	11·7	13·4	10·8	1·27	0·443
Brucella spp.	3·2	5·2	2·2	2·40	0·121
N. caninum	17·2	29·8	10·8	3·50	<0·001
N. caninum and/or *Brucella* spp. and/or BVDV	28·8	42·6	22·0	2·62	<0·001
N. caninum and *Brucella* spp.	1·0	2·2	0·4	6·11	0·118
Brucella spp. and BVDV	0·5	0·7	0·4	2·00	0·623
N. caninum and BVDV	1·7	3·0	1·1	2·71	0·195
Brucella spp. and *N. caninum* and BVDV	—	—	—	—	—

OR, Odds ratio; BVDV, Bovine viral diarrhoea virus.
* *P* values (comparing cases and controls) and associated odds ratios are from univariable logistic regression.

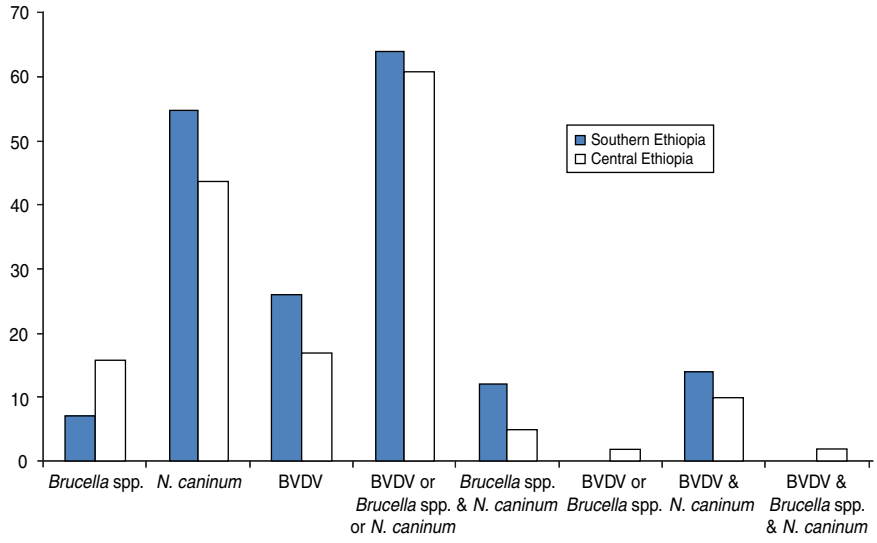

Fig. 2 [*colour online*]. Farm-level proportions of seroreactors to bovine viral diarrhoea virus (BVDV), *Brucella* spp., *Neospora caninum* and mixed infection in central and southern Ethiopia.

were often seropositive for antibodies to *N. caninum*, less frequently to BVDV, and rarely to *Brucella* spp. Although the sensitivity and specificity of the tests used for detecting the antibodies of each of these pathogens are not identical, the tests are sufficiently robust for us to believe that comparison of the results provides an accurate reflection of the actual situation. *Neospora* seropositivity was clearly associated with abortion or stillbirth whereas BVDV or *Brucella* were not. As presence of antibodies to *N. caninum* is indicative of infection, our finding suggests that infection with *N. caninum* was the probable cause of abortion in the studied farms, and indicates that the emphasis on brucellosis being the major cause of abortion/stillbirth in dairy cattle in Ethiopia is probably erroneous. This association between *N. caninum* seropositivity and abortion or stillbirth is in agreement with many previous reports from other countries [6, 14, 15, 18, 26, 27].

As the causes of abortion/stillbirth are multifactorial, exposure of cattle to any one of the three agents investigated may be responsible for this outcome, as illustrated in Figure 3. Thus, the reasons for abortion/stillbirth in cattle should be considered as broadly as possible (both infectious and non-infectious), and the differential list of infectious agents

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Table 2. *Reproductive disorders other than stillbirth and abortion in relation to serological profiles in cases (history of stillbirth and/or abortion) and controls*

Reproductive disorder	Level	Cases (*n*)	Controls (*n*)	BVDV (*P* value)*	*Brucella* spp. (*P* value)*	*N. caninum* (*P* value)*
Prolonged calving interval†	No	4	109			
	Yes	63	49	0·568	0·411	0·005
Retained fetal membranes	No	42	177			
	Yes	92	60	0·343	0·836	0·001
Uterine infection	No	37	171			
	Yes	97	66	0·484	0·686	0·001
Delivery of weak calf	No	120	217			
	Yes	14	20	0·510	0·436	0·982
Delivery of congenitally defective calf	No	122	220			
	Yes	12	17	0·161	—	0·283
Dystocia	No	126	232			
	Yes	8	5	0·650	—	0·613
Uterine or vaginal prolapse	No	128	225			
	Yes	6	12	0·575	—	0·615
Repeat breeding‡	No	119	211			
	Yes	15	26	0·339	0·031	0·760

BVDV, Bovine viral diarrhoea virus.
* Associated *P* values are from univariable logistic regression.
† Prolonged calving interval ⩾18 months.
‡ Repeat breeding = cow served more than three times per pregnancy.

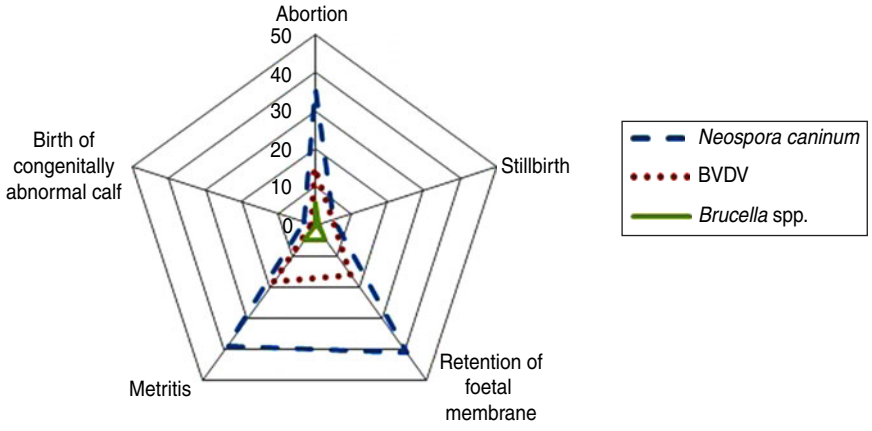

Fig. 3 [*colour online*]. Frequency of reported clinical disorders in cases in relation to the infectious agent's serostatus profile. BVDV, Bovine viral diarrhoea virus.

should consider all possible reasons, including *Neospora*, as well as the other agents investigated in this study, and also additional agents such as bovine herpesvirus-1 and *Leptospira* spp.

In considering seropositivity to more than one of the infectious agents included in the study, <4% of the animals had evidence of exposure to two of the infectious agents (<2% per pair of infectious agents), presumably due to the relatively low seroprevalence of both BVDV and *Brucella* spp. No animals in this study were seropositive to all three study agents. These findings differ from the findings of de Melo *et al.* [28] in Brazil who observed higher co-existence of *N. caninum* seropositivity with antibodies against BVDV and also bovine herpesvirus. This could be an epidemiological issue that needs further investigation, particularly regarding whether any other infectious agents or other factors, such as stress factors, act as concomitant agents triggering abortion in animals that have been exposed to any of these infectious agents but do no experience reproductive problems.

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Table 3. *Associations between covariates of abortion and/or stillbirth with seropositivity to BVDV,* Brucella *spp., and* Neospora caninum *in breeding and commercial dairy cattle*

Covariates	Level	Cases (*n*)	Controls (*n*)	BVDV (*P* value)	*Brucella* spp. (*P* value)*	*Neospora caninum* (*P* value)*	Abortion and/or stillbirth (*P* value)*
Age (months)	Calf (6–12)	0	28	—		—	—
	Young (>12–18)	0	12	0·538	—	0·819	—
	Heifer (>18–36)	13	28	0·522	—	0·465	—
	Adult (>36)	121	200	0·174	—	0·333	0·981
Breed	Friesian cross	10	17				
	Friesian	113	231	0·321	—	0·168	0·137
	Jersey	11	20	0·090	—	0·498	0·658
Parity	No	—	40				
	Primiparous	13	39	0·970	0·377	0·740	0·042
	Pluriparous	121	189	0·896	0·877	0·582	<0·001
Origin	Homebred	53	142				
	Purchased	81	126	0·057	0·863	<0·001	0·297
Maternal abortion/ stillbirth history	No	40	120				
	Yes	13	22	0·057	0·863	0·030	0·063
	Unknown	81	126	0·172	0·829	<0·001	0·113

BVDV, Bovine viral diarrhoea virus.
* Associated *P* values are from univariable logistic regression.

Table 4. *Multivariable logistic regression estimate of* Neospora caninum *exposure risk at individual animal level for animals with a history of abortion and/or stillbirth*

Variables	Level	OR*	(95% CI)	*P* value
Origin	Homebred *vs.* purchased	3·9	(2·01–7·66)	<0·001
Maternal abortion/stillbirth history	Yes *vs.* no	3·1	(1·11–8·51)	0·030

OR, Odds ratio; CI, confidence interval.
* Odds ratios measured by a random-effect (herd) logistic regression.

Table 5. *Associations between* Neospora caninum *and reproductive disorders measured by a random-effect* (*herd*) *logistic regression*

Reproductive disorders	Level	OR	(95% CI)	*P* value
Extended calving interval	Yes *vs.* no	2·56	(1·27–5·15)	0·008
Abortion	Yes *vs.* no	3·04	(1·74–5·30)	<0·001
Retention of fetal membranes	Yes *vs.* no	2·40	(1·37–4·22)	0·002
Uterine infection	Yes *vs.* no	2·45	(1·41–4·24)	0·001
Status (abortion or stillbirth)	Control, case	3·45	(1·87–6·37)	<0·001

OR, Odds ratio; CI, confidence interval.

Extended calving interval, abortion, retention of fetal membranes and uterine infection were more frequently reported in *Neospora*-seropositive animals than seronegatives animalss. These disorders have previously been suggested to be the possible consequences of neosporosis [29–31], and our findings support these suggestions.

N. caninum and BVDV infection may cause pregnancy loss throughout gestation [32, 33], including during the first trimester. If a conceptus dies *in utero* within the first 3 months, the cow may return to heat again before it has been identified as being pregnant [34–36]. In these instances, the cattle in this study may have been classified as controls (non-aborting). Thus,

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the frequency of abortion may have been underestimated in our study. We suggest that further studies focusing on late embryonic and early fetal mortality would be useful in providing a better understanding of the impact of these pathogens on the reproductive and economic performance of Ethiopian dairy cattle.

The association of individuals whose dam had an abortion or stillbirth with seropositivity for *N. caninum*, probably demonstrates the importance of vertical transmission in maintaining the infection as evidenced by Hall *et al*. [37] and Haddad *et al*. [38]. Similarly, the increased frequency of seropositive animals in purchased animals, rather than homebred, could also reflect incorrect culling practices (failure to remove defective animals from breeding line). We assume this to be due to a lack of awareness on the necessity of appropriate culling practises.

Demographic factors like age, breed, parity had no apparent effect on serological profile. Some previous studies have also found no effect from breed or age, e.g. studies from Kartum and Gazira state in Sudan [20] and Mashhad, Iran [39], while other reports indicate that serostatus is affected by particular breeds [19, 40]. As production system, rather than breed [6] may be the determining factor, it is important that results are interpreted with caution.

In conclusion, the results of our study suggest that neosporosis is a significantly more important cause of abortion and/or stillbirth in breeding and dairy farms of southern and central Ethiopia than BVDV and brucellosis. Cows seropositive to *N. caninum* had a substantially higher risk of having suffered abortion or stillbirth relative to their seronegative counterparts. Furthermore, these animals frequently suffered uterine infection, retention of fetal membranes and had prolonged calving intervals. We suggest that neosporosis is a significant hindrance to the development of the dairy industry in Ethiopia as this infection results in considerable fetal losses. The need to investigate the status and impact of infectious agents on the dairy industry at a larger scale is imperative, and future studies should investigate approaches to breeding stack replacement and design and evaluation of appropriate control strategies.

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DECLARATION OF INTEREST

None.

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