Original Article
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Dembo polymerase chain reaction technique for detection of bovine abortion, diarrhea, and respiratory disease complex infectious agents in potential vectors and reservoirs

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Bovine abortion, diarrhea, and respiratory disease complexes, caused by infectious agents, result in high and significant economic losses for the cattle industry. These pathogens are likely transmitted by various vectors and reservoirs including insects, birds, and rodents. However, experimental data supporting this possibility are scarce. We collected 117 samples and screened them for 44 bovine abortive, diarrheal, and respiratory disease complex pathogens by using Dembo polymerase chain reaction (PCR), which is based on TaqMan real-time PCR. Fifty-seven samples were positive for at least one pathogen, including bovine viral diarrhea virus, bovine enterovirus, *Salmonella enterica* ser. Dublin, *Salmonella enterica* ser. Typhimurium, and *Neospora caninum*; some samples were positive for multiple pathogens. Bovine viral diarrhea virus and bovine enterovirus were the most frequently detected pathogens, especially in flies, suggesting an important role of flies in the transmission of these viruses. Additionally, we detected the *N. caninum* genome from a cockroach sample for the first time. Our data suggest that insects (particularly flies), birds, and rodents are potential vectors and reservoirs of abortion, diarrhea, and respiratory infectious agents, and that they may transmit more than one pathogen at the same time.

Keywords: Dembo polymerase chain reaction, cattle, disease reservoirs, disease vectors, virulence factors

Introduction

Abortion, diarrhea, and respiratory infectious agents cause a broad spectrum of diseases, resulting in significant economic losses in the cattle industry [6,9,19]. In the USA, it has been estimated that late-term cattle abortions cost between US dollar (USD) 500 and USD 900 per case and that the cattle respiratory disease complex causes 70% to 80% of all feedlot cattle morbidity and 40% to 50% of all cattle mortality, resulting in major economic losses amounting to more than USD 500 million per year [10,19]. Diarrhea in cattle decreases fertility and productivity, including reductions in milk production and weight gain [9,11]. According to the United States Department of Agriculture's reports, 57% of deaths of weaning calves in the USA were due to diarrhea [31].

Various vectors and reservoirs have important roles in the

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transmission of pathogens [4,22,27]. Vector-borne diseases are transmitted by insects, such as mosquitoes, flies, ticks, fleas, and lice [22,27]. Vectors may be divided into two types: biological and mechanical. Biological vectors carry infectious agents or pathogens within their bodies, where the infectious agents undergo multiplication and/or development, consequently transmitting the infectious agents to the host through bites. Mosquitoes are a biological vector of many pathogens. Mechanical vectors transfer pathogens from an infected host or a contaminated substrate to a susceptible host without multiplying and/or developing of the pathogens within the vector. Many insects can serve as mechanical vectors [27]. Reservoirs are one or more epidemiologically connected populations or environments, in which the infectious agent can be permanently maintained, and from which infection is transmitted to the defined target population [8]. Mammals, such as rodents and carnivores, are examples of most commonly known disease reservoirs [8].

Several studies have shown that vectors and reservoirs play a critical role in the transmission of a broad spectrum of pathogens, including bovine viral diarrhea virus (BVDV), bovine enterovirus (BEV), *Salmonella enterica* ser. Typhimurium, *Escherichia coli*, and *Campylobacter* spp. [3,7,22,27].

Previously, we developed detection systems for 19 bovine diarrheal agents and 16 bovine respiratory disease complexes by using a detection system of microbes for bovine (Dembo) diarrheal diseases via real-time polymerase chain reaction (Dembo diarrhea-PCR) and a similar system for bovine respiratory disease complex via real-time PCR (Dembo respiratory-PCR), respectively; both detection systems are based on TaqMan real-time PCR [13,31]. The Dembo-PCR method exhibits high sensitivity, high specificity, rapidity, and a capacity to simultaneously detect all targeted infectious agents.

In the present study, we developed a real-time PCR-based system for detection of 24 bovine abortive agents (Dembo abortion-PCR). Subsequently, by using Dembo-PCR, we evaluated whether infectious agents causing diseases in cattle could be transferred by vectors and reservoirs such as insects, rodents, and birds.

Materials and Methods

Primer and probe design

We selected 24 pathogens as bovine abortive agents. To detect 15 of those pathogens, we used previously reported primers and probes [18,20,23-25,28,29,31-33,36]. We used newly designed primers and probes for the remaining 9 pathogens: Schmallenberg virus, Chuzan virus, Sathuperi virus, Shamonda virus, Douglas virus, Ibaraki virus, Aino virus, *Toxoplasma gondii*, and *Neospora caninum*. The multiple nucleotide sequences for each of these 9 pathogens were obtained from the National Center for Biotechnology Information database (Supplementary Table 1), and new sets of primers and probes were designed by using PrimerQuest tool (Integrated DNA Technologies, USA) based upon a consensus sequence acquired by multiple alignments of the obtained sequences obtained via the BioEdit software 7.0.5 (Ibis Therapeutics, USA). Bovine β-actin was used as the internal control for the extraction of nucleic acids [13,31,34]. All probes at the 5′ end were indicated by the dye FAM (6-carboxyfluorescein) and by the fluorescent dye TAMRA (6-carboxytetramethylrhodamine) at the 3′ end. All primers and probes were purchased from Sigma-Aldrich (USA) or Integrated DNA Technologies. Table 1 lists the primers and probes used in this study.

Extraction of nucleic acids

Viral nucleic acids were extracted by using High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Germany). The QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) was used according to the manufacturer's instructions to obtain bacterial, protozoal, and fungal DNA. The extracted DNA and RNA were stored at −80°C until use.

Real-time PCR amplification

All TaqMan real-time PCR assays were performed under the same reaction conditions used for Dembo diarrhea-PCR and Dembo respiratory-PCR [13,31]. A One Step PrimeScript RT-PCR Kit (Perfect Real Time; TaKaRa Bio, Japan) was used to detect viral RNA, and Premix Ex Taq (Perfect Real Time) was used to detect the viral, protozoal, fungal, and bacterial DNAs. The real-time PCR assay was performed with the Applied Biosystems 7300 Real-Time PCR System (ABI 7300; Applied Biosystems, USA) for screening and with the LightCycler Nano (Roche Diagnostics) for validation of positive samples detected during screening. To analyze the fluorescence data, the automatic quantification algorithm was used in LightCycler Nano Software 1.1 and Applied Biosystems 7300 Real-Time PCR software. The parameters of analysis were as follows: Exclude early cycle = 7, minimum relative amplifications = 0, and minimum amplification quality = 5.

Validation of the Dembo abortion-PCR

To evaluate the sensitivity of the Dembo abortion-PCR, the synthetic DNA (including target genome regions) of all target pathogens was used to determine the limit of detection (LOD), correlation coefficient (R^2), and PCR efficiency (E). The synthetic DNA was fabricated at Integrated DNA Technologies. After creation of standard curves, LOD, R^2, and E were calculated as described previously [13,31]. To validate the specificity of the Dembo abortion-PCR, 22 cattle blood samples and 14 cattle aborted fetus spinal cord samples were collected and subjected to Dembo abortion-PCR. All samples were negative for the presence of target pathogens; no positive or false positive

Table 1. Information on all primers and probes used in current study

Target pathogen	Target gene		Primer/probe sequence 5′–3′ (FAM/TAMRA)	Reference
Aino virus	M polyprotein	F	AGCAAATCCCATTGCGTGA	This study
		R	CAGACTTCTGCTGGCACATTA	
		P	AGGGACAACTGGCTCTCGCT	
Akabane virus	S segment	F	TCAACCAGAAGAAGGCCAAGAT	[28]
		R	GGGAAAATGGTTATTAACCACTGTAAA	
		P	TTACATAAGACGCCACAACCA	
Bluetongue virus	NS3 gene	F	AAATMTTGGAYAAAGCRATGTCAAA	[33]
		R	CTYACRTCATCACGAAACGCT	
		P	AARGCTGCATTCGCATCGTACGC	
Chuzan virus	VP7 gene	F	TGATCGAACGCCAACACTT	This study
		R	GGCAATCCAACCCTCATACA	
		P	TATCACCACAATGGCATGCATTGCG	
Ibaraki virus	Vp3 gene	F	TACAGCGGGACCTAGGTTTA	This study
		R	GTTCTCCCGTTGGACCATATT	
		P	TGGCACGACAGCTTGATATTGCCT	
Simbu group*	N, NSs gene, S segment	F	TGAAGATGTACCACAACGGAAT	This study
		R	GAGGAAGAAGACTCTAGCAACAC	
		P	ACCTCCGGGTTAAATGTAGCTGC	
Aspergillus spp.	18S ribosomal RNA gene	F	GCCCGCCGTTTCGAC	[18]
		R	CCGTTGTTGAAAGTTTTAACTGATTAC	
		P	CCCGCCGAAGACCCCAACATG	
Brucella abortus	omp2a gene	F	GCGGCTTTTCTATCACGGTATTC	[24]
		R	CATGCGCTATGATCTGGTTACG	
		P	CGCTCATGCTCGCCAGACTTCAATG	
Campylobacter fetus subsp. *venerealis*	*nahE* gene	F	TTCAAAAGCTCTTGGGGTTAC	[32]
		R	AAAGCCTTGTTTAGAACAATATAACTC	
		P	ACTCGTGGTGGAGAGCGTAG	
Chlamydophila abortus	ompA gene	F	GCAACTGACACTAAGTCGGCTACA	[23]
		R	ACAAGCATGTTCAATCGATAAGAGA	
		P	TAAATACCACGAATGGCAAGTTGGTTTAGCG	
Leptospira spp.	lipL32 gene	F	AAGCATTACCGCTTGTGGTG	[29]
		R	GAACTCCCATTTCAGCGAT	
		P	AAAGCCAGGACAAGCGCCG	
Listeria monocytogenes	iap gene	F	CATGGCACCACCAGCATCT	[25]
		R	ATCCGCGTGTTTCTTTTCGA	
		P	CGCCTGCAAGTCCTAAGACGCCA	
Neospora caninum	NC5 gene	F	GGGATACGTGGTTTGTGGTTAG	This study
		R	CACAGAACACTGAACTCTCGATAAG	
		P	TCACGTTGAAATCAGCCTGCGTCA	
Sarcocystis cruzi	18S ribosomal RNA gene	F	TCTGCTGGAAGCAATCAGTC	[20]
		R	TTGAAGCAGGCTTATTGCCT	
		P	ACCCATCTATATTGGGATAATACCGTTACT	
Toxoplasma gondii	p30 gene	F	GCCTCATCGGTCGTCAATAA	This study
		R	GTCATTGTAGTGGGTCCTTCC	
		P	AGCACTCTTGGTCCTGTCAAGTTGT	
Tritrichomonas foetus	5.8S ribosomal RNA gene	F	GCGGCTGGATTAGCTTTCTTT	[36]
		R	GGCGCGCAATGTGCAT	
		P	ACAAGTTCGATCTTTG	
β-ACTIN	Actin	F	AGCGCAAGTACTCCGTGTG	[34]
		R	CGGACTCATCGTACTCCTGCTT	
		P	TCGCTGTCCACCTTCCAGCAGATGT	
Dembo diarrhea primers/probes				[31]
Dembo respiratory primers/probes				[13]

F, forward primer; R, reverse primer; P, probe. *Simbu group: Douglas virus, Sathuperi virus, Shamonda virus, Schmallenberg virus.

results were obtained.

Analysis of field samples

A total of 117 vector and reservoir samples, including 63 flies, 18 gadflies, 7 insects, 14 fecal and intestinal contents from rodents, and 14 fecal samples from birds were collected from inside and outside of 4 dairy cattle farms and 17 beef cattle farms between 2014 to 2016 in Japan (Supplementary Table 2). Nucleic acids were extracted from each sample. To identify bovine diarrheal pathogens, all 117 samples were screened individually. To detect bovine abortive and respiratory disease complex pathogens, extracted nucleic acid samples were pooled as shown in Table 2. After sample pooling, RNAs in each pooled sample were reverse transcribed into complementary DNA (cDNA) by using SuperScript III Reverse Transcriptase (Invitrogen, USA), and then, the cDNA and genomic DNA were amplified by using GenomiPhi V2 DNA Amplification Kit (GE Healthcare, UK). The extracted nucleic acids were evaluated in triplicate by targeting abortive, diarrheal, and respiratory disease complex pathogens in a single run of Dembo-PCR [31]. When the Cq values were calculated by algorithm described above in more than two out of three runs,

Table 2. Pooling method used for the extracted nucleic acids obtained from vectors samples

Type of sample	Samples	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Pool 6	Pool 7	Pool 8	Pool 9	Pool 10
Fly	64	9	9	9	9	9	9	9	9	9	10
Gadfly	18	9	9								
Feces of rodent	14	7	7								
Feces of birds	14	7	7								
Insects	7	7									

Data are presented as number of samples.

Table 3. Results of sensitivity tests for bovine abortive pathogens obtained by using the LightCycler Nano (Roche Diagnostics)

Target pathogen	LOD (copies/reaction)		E		R^2	
	1	2	1	2	1	2
Sarcocystis cruzi	1	1	2.026	1.884	0.9827	0.9981
Toxoplasma gondii	10	10	2.048	2.235	0.9984	0.9814
Tritrichomonas fetus	10	10	1.992	1.838	0.9981	0.9738
Neospora caninum	10	100	1.954	1.972	0.9998	0.9679
Campylobacter fetus	10	100	1.909	2.120	0.9991	0.9634
Chlamydophila abortus	1	10	2.031	1.883	0.9582	0.9999
Listeria monocytogenes	10	10	1.941	1.940	0.9994	0.9997
Leptospira spp.	100	10	1.932	1.935	0.9950	0.9996
Brucella abortus	1	10	1.957	2.017	0.9994	0.9998
Bluetongue virus	1	10	2.076	1.987	1	0.9993
Akabane virus	100	100	1.891	1.858	0.9976	0.9993
Aino virus	10	10	1.975	1.878	0.9989	0.9971
Chuzan virus	10	10	1.938	1.944	0.9986	0.9998
Bovine herpes virus-1	100	10	2.159	1.930	0.6052	0.9948
Bovine viral diarrhea virus	100	100	1.930	2.007	0.9927	0.9897
Simbu group*	10	10	1.842	1.863	0.9981	0.9998
Ibaraki virus	10	10	1.921	1.944	0.9993	0.9980
Aspergillus spp.	1	1	2.130	2.080	0.9170	0.9900
Salmonella Enteritidis	10	10	1.962	2.159	0.9980	0.9550
Salmonella Typhimurium	100	100	2.192	2.173	0.9950	0.9960
Salmonella Dublin	10	10	1.958	1.930	0.9850	0.9910

LOD, limit of detection; E, polymerase chain reaction efficiency; R^2, coefficient of determination; 1, first reaction; 2, second reaction. *Simbu group: Douglas virus, Sathuperi virus, Shamonda virus, Schmallenberg virus.

the samples were considered positive.

Results

Sensitivity and specificity of the Dembo abortion-PCR

Table 3 shows the LOD, R^2, and E value of the Dembo abortion-PCR results from the LightCycler Nano instrument. The LOD, according to the DNA copy number of all pathogens, was between 1 and 100 copies per reaction. The coverage of the calibration curves for each assay was within a linear dynamic range of more than five orders of magnitude, and R^2 values were at least 0.9582. The E values were in the range of 92.1% to 106%. When using the ABI 7300 instrument, the LODs of the Dembo respiratory- and abortion-PCR assays were evaluated with 100 copies per reaction. When the sensitivity was lower than 100 copies per reaction, lower diluents were used to evaluate the LOD. All sets of primers and probes, except for 5, showed sensitivities of 100 copies per reaction (Table 4).

Analysis of field samples via Dembo-PCR

Field samples were analyzed via two different approaches. To detect diarrheal pathogens, all 117 samples were individually screened via Dembo-PCR using the LightCycler Nano instrument. Fifty-seven of the 117 samples were positive for at least one diarrheal pathogen; 34 of the 63 flies (53.97%), 8 of the 14 fecal and intestinal contents from rodents (57.14%), 8 of the 18 gadflies (44.44%), 5 of the 14 fecal samples from birds (35.71%), and 2 of the 7 insects (28.57%) were positive for at least one pathogen including BVDV, BEV, *S. enterica* ser. Dublin, and *S. enterica* ser. Typhimurium. To detect abortive and respiratory disease complex pathogens, 15 pooled samples were screened via Dembo-PCR using the ABI 7300 instrument. *N. caninum* was detected only in an insect pooled sample, which consisted of 7 different insect samples including 2 cockroaches, 2 spiders, and 3 unidentified insects. All other pathogen results were negative in all of the pooled samples. To determine which insects were positive for *N. caninum*, each of the 7 insect samples was analyzed using the LightCycler Nano instrument, resulting in the cockroach sample testing positive exclusively. Table 5 summarizes the numbers of positive samples from each vector and reservoir.

Discussion

This is the first study that simultaneously evaluated the presence of a wide range of bovine abortion pathogens in potential vectors and reservoirs by using the Dembo abortion-PCR, a highly sensitive and rapid pathogen detection system. The Dembo abortion-PCR was performed by using the same reaction conditions as those reported for Dembo diarrhea-PCR and Dembo respiratory-PCR [13,31]. We first used the Dembo abortion-PCR to detect 24 cattle abortive agents including 11 viruses, 8 bacteria, 4 protozoa, and 1 fungus. Subsequently, 44 bovine abortive, diarrheal, and respiratory disease complex pathogens, including 23 viruses, 12 bacteria, 6 protozoa, 2 mycoplasmas, and 1 fungus, were targeted in a single run by Dembo-PCR. For the Dembo abortion-PCR, additional primers and probes were designed to detect the Schmallenberg virus, Sathuperi virus, Douglas virus, and Shamonda virus [35]. These

Table 4. Results for the LOD of bovine abortive and respiratory disease complex pathogens obtained by using the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems)

Target pathogen	LOD (copies/reaction)
Bovine viral diarrheal virus	100
Bovine coronavirus	100
Mammalian orthoreovirus	100
Bovine herpes virus-1	100
Salmonella Dublin	100
Salmonella Enteritidis	100
Salmonella Typhimurium	100
Bovine adenovirus 3	100
Bovine parainfluenza virus 3	100
Bovine respiratory syncytial virus	100
Influenza D virus	100
Bovine rhinitis A virus	100
Bovine rhinitis B virus	100
Bovine adenovirus 7	100
Mannheimia haemolytica	100
Pasteurella multocida	250
Histophillus somni	250
Trueperella pyogenes	250
Mycoplasma bovis	250
Ureaplasma diversum	250
Bluetongue virus	100
Akabane virus	100
Chuzan virus	100
Aino virus	100
Ibaraki virus	100
Simbu group*	100
Chlamydophila abortus	100
Neospora caninum	100
Campylobacter fetus subsp. *venerealis*	100
Toxoplasma gondii	100
Sarcocystis cruzi	100
Brucella abortus	100
Tritrichomonas foetus	100
Listeria monocytogenes	100
Aspergillus spp.	100
Leptospira spp.	100

LOD, limit of detection; PCR, polymerase chain reaction. *Simbu group: Douglas virus, Sathuperi virus, Shamonda virus, Schmallenberg virus.

Table 5. Positive results obtained from clinical samples by using Dembo-PCR assay

Type of sample	BVDV	BEV	*S*. Dublin	BVDV + *S*. Dublin	BVDV + BEV	BVDV + *S*. Typhimurium	BEV + *S*. Dublin	BEV + *N. caninum**
Fly	5	23	1	0	3	0	2	0
Feces of rodent	3	0	1	1	0	1	0	0
Contents in intestine of rodent	0	1	1	0	0	0	0	0
Feces of bird	3	1	0	0	0	0	1	0
Gadfly	1	1	4	1	1	0	0	0
Unidentified insect	1	0	0	0	0	0	0	0
Cockroach	0	0	0	0	0	0	0	1

PCR, polymerase chain reaction; BVDV, bovine viral diarrhea; BEV, bovine enterovirus; *S*. Dublin, *Salmonella enterica* ser. Dublin; *S*. Typhimurium, *Salmonella enterica* ser. Typhimurium; *N. caninum*, *Neospora caninum*. **N. caninum* was confirmed by nested PCR (data not shown).

viruses belong to the Simbu group, which includes important viruses causing abortion in cattle [35].

BVDV was one of the most frequently detected pathogens from the wide spectrum of examined vectors and reservoirs, including flies, gadflies, and rodent and bird fecal matter. It should be noted that in addition to diarrhea, BVDV causes abortion and respiratory diseases [13,17,31]. Out of the 117 vector and reservoir samples, 20 tested positive for BVDV, and 8 flies that were positive for BVDV formed the largest group among the examined vectors and reservoirs. Our results are consistent with those in a previous study that reported flies as a potential source of BVDV transmission in cattle [3]. We also detected BVDV in rodent and avian fecal matter; in contrast, no previous study has reported the presence of BVDV in rodents and birds. The results of these studies (both current and past [13,31]) imply that BVDV is one of the most important infectious agents in cattle-related diseases and that BVDV could be transmitted by flies, rodents, and birds.

BEV was positive in 34 samples, including 28 flies, 2 gadflies, 1 rodent fecal matter sample, 2 avian fecal matter samples, and 1 cockroach. BEV, a common virus in the environment, is very stable under a broad range of environmental conditions such as pH, temperature, and salinity. These physiological properties of BEV facilitate easy transmission of BEV to cattle [15]. Although BEV was detected from diarrheal samples in this study, it also has the potential to cause abortion in cattle [31]. While previous studies have mentioned that BEV could be spread in the environment and contaminate water and food, our present study represents the first detection of BEV in vectors and reservoirs [12].

Both *S. enterica* ser. Dublin and *S. enterica* ser. Typhimurium were detected in flies, gadflies, and fecal samples of rodents and birds, suggesting that those animals serve as reservoirs [1,21,22,27]. In cattle, these two serovars of *Salmonella* cause diarrhea and may also cause abortion and respiratory disease [2,13,31]. In this study, we did not demonstrate whether cattle inside the farms were infected with these pathogens. Further study is needed to isolate these bacteria from potential vectors and reservoirs because we detected only the bacterial genomes in this study; additionally, there is a need to investigate transmission from these pests to cattle.

N. caninum and BEV were simultaneously detected in one cockroach sample. *N. caninum* is an obligate intracellular coccidian parasite that is globally distributed and is one of the major pathogens causing abortion in cattle [16,30]. A broad spectrum of wild and domestic animals can be infected by *N. caninum* with dogs, coyotes, and gray wolves (*Canis lupus*) considered to be final hosts of *N. caninum*. However, mammals and birds, including cattle, sheep, goat, water buffalo, horse, donkey, bison, white-tailed deer, red fox, chicken, pigeon, sparrow, feral swine, capybara, and rabbit can also serve as potential natural intermediate hosts for this pathogen. Although *N. caninum* has been detected in several mammals and birds, further investigation into the lifecycle and hosts of this pathogen is required [5,16,26,30]. Cockroaches are vectors or potential transmitters of protozoans such as *T. gondii*, *Sarcocystis* oocysts, and others [14,27,37]. Our study detected the *N. caninum* genome in a cockroach sample for the first time, implying that cockroaches may play a role in its life cycle. Alternatively, cockroaches may serve as a potential vector of *N. caninum*.

Our results have shown that insects and rodents, while acting as potential vectors and reservoirs of cattle pathogens, can carry more than one pathogen at the same time. This is the first demonstration of vectors and reservoirs acting in tandem to transmit more than one infectious agent.

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Conflict of Interest

The authors declare no conflicts of interest.

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Supplementary Table 1. GenBank accession numbers for the reference sequences used for primer and probe design

Pathogen name	GenBank accession No.
Aino virus	HE795088, AB542973, AB542972, AB542971, AB542970, AB542969, AB542978, AB542967, AB542966, AB100695
Chuzan virus	NC005988, AB014727, AY078469, AY078470
Ibaraki virus	AB107801, AB106908, AB106907, AB106903, AB106902, AB106901, AB106900
Simbu group*	KC108864, NC018464, NC018462, HE795092
Toxoplasma gondii	S63900, DQ872518, GQ253080, GQ253086, DQ872517, AK317969, GQ253075, GQ253073, X14080, AY187278, JX045363, JX045390, JX045394, HM776940, JX045356, XM002368164, S73634, AY217784, AY661791
Neospora caninum	KX683874, KX683873, KU253799, KR106184, KR106181, KP715562, KP715561, KP715560, KP715559, KF649845, KF649845, KF649846, KF649847, HM031965

*Simbu group: Douglas virus, Sathuperi virus, Shamonda virus, Schmallenberg virus.

Supplementary Table 2. Summary of information about the samples included in this study

Farm identifier	Type of farm	Inside/outside of farm	Type of sample	No. of samples
A	Dairy	Outside	Gadfly	6
		Inside	Spider	1
		Inside	Mosquito	3
		Inside	Fly	14
		Outside	Feces of Bird	7
		Inside	Intestine contents of rodent	1
B	Dairy	Inside	Fly	14
		Inside	Cockroach	1
		Outside	Gadfly	2
		Inside	Gadfly	2
		Inside	Feces of Bird	5
		Outside	Feces of Bird	2
		Inside	Intestine contents of rodent	1
C	Dairy	Inside	Fly	2
D	Dairy	Inside	Fly	1
E	Meat	Inside	Fly	2
		Inside	Spider	1
		Inside	Unidentified insect	1
		Outside	Gadfly	2
		Outside	Feces of rodent	2
		Inside	Feces of rodent	2
F	Meat	Inside	Fly	2
		Outside	Gadfly	2
		Outside	Fly	2
G	Meat	Inside	Fly	1
		Outside	Fly	1
		Inside	Feces of rodent	1
		No information	Feces of rodent	1
H	Meat	Inside	Fly	1
		Outside	Fly	1
		Outside	Gadfly	3
I	Meat	Inside	Fly	1
		Outside	Fly	1
J	Meat	Inside	Fly	2
		Outside	Fly	1
K	Meat	Inside	Fly	1
		Outside	Fly	1
L	Meat	Inside	Fly	1
M	Meat	Inside	Fly	2
		Inside	Feces of rodent	1
		Outside	Feces of rodent	2
		Inside	Gadfly	1
N	Meat	Inside	Fly	1
O	Meat	Inside	Fly	1
P	Meat	Inside	Fly	1
		Outside	Fly	1
Q	Meat	Inside	Fly	2
R	Meat	Inside	Fly	1
		Outside	Fly	1
		No information	Feces of rodent	2
S	Meat	Inside	Fly	1
T	Meat	Inside	Fly	1
		Outside	Fly	1
U	Meat	Inside	Fly	1
		Outside	Fly	1
		Inside	Feces of rodent	1