Journal of Veterinary Diagnostic Investigation

Simultaneous detection of *Chlamydia* spp., *Coxiella burnetii*, and *Neospora caninum* in abortion material of ruminants by multiplex real-time polymerase chain reaction

Kerstin Reisberg, Abdelfattah M. Selim and Wolfgang Gaede

Published by:

$SAGE

http://www.sagepublications.com

On behalf of:

AAVLD

Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc.

Additional services and information for *Journal of Veterinary Diagnostic Investigation* can be found at:

Email Alerts: http://vdi.sagepub.com/cgi/alerts

Subscriptions: http://vdi.sagepub.com/subscriptions

Reprints: http://www.sagepub.com/journalsReprints.nav

Permissions: http://www.sagepub.com/journalsPermissions.nav

>> Version of Record - Sep 12, 2013

OnlineFirst Version of Record - Aug 13, 2013

What is This?

Simultaneous detection of *Chlamydia* spp., *Coxiella burnetii*, and *Neospora caninum* in abortion material of ruminants by multiplex real-time polymerase chain reaction

Kerstin Reisberg, Abdelfattah M. Selim, Wolfgang Gaede[1]

Abstract. *Chlamydia* spp., *Coxiella burnetii*, and *Neospora caninum* are responsible for reproductive diseases and are closely linked with high abortion rates in ruminants. Furthermore, *C. burnetii* and *Chlamydia* spp. have zoonotic potential. A real-time polymerase chain reaction (PCR) assay was developed for the simultaneous detection of *Chlamydia* spp., *C. burnetii*, and *N. caninum*. The detection of beta-actin as internal control in the same PCR reaction provides additional information about sample quality by detecting the presence of PCR inhibitors. The multiplex real-time PCR developed in the current study shows a greater sensitivity compared to previously used single-target PCR reactions with a reproducible detection limit of 0.13 plasmid copies per PCR for each target. Additional parallel amplification of all detectable pathogens did not adversely impact sensitivity. This new multiplex PCR allows the highly sensitive, cost-effective, and rapid detection of 3 important pathogens and has the potential to be a useful time-saving tool in the routine diagnosis of abortion cases in ruminants.

Key words: Abortion; multiplex real-time polymerase chain reaction; pathogens; ruminants.

Abortion in ruminants is a common economic problem worldwide,[16,19] with *Neospora caninum* causing an estimated annual loss of $546.3 million in the U.S. dairy industry alone.[16] Three significant infectious agents causing abortion in ruminants are *Chlamydia* spp.,[4,13] *Coxiella burnetii*, and *Neospora caninum*.[1,12] In addition to impairing animal health, *C. burnetii* and several members of the genus *Chlamydia* are responsible for zoonotic diseases such as abortions in human beings.[10,13] The enormous risk potential of *C. burnetii* being spread by goats was shown by the Dutch outbreak of 2007–2010, which caused more than 4,000 human infections.[6]

Because these 3 pathogens cause similar symptoms, such as abortion, stillbirth, and the delivery of weak offspring,[7,12] diagnostic investigations of ruminant abortions should include at least these pathogens. For rapid and specific diagnosis, direct detection by polymerase chain reaction (PCR) has been proven to be an important tool.[5,11,14] To better use diagnostic resources, the aim of the current study was to develop a multiplex real-time PCR for the simultaneous and rapid detection of *Chlamydia* spp., *C. burnetii*, and *N. caninum*.

For the multiplex real-time PCR developed in the current study, previously described probe and primers for *Chlamydia* spp.[14] and *N. caninum* were used.[5] For *C. burnetii*, a previously described assay that targets the iso citrate dehydrogenase gene (*icd*)[11] was used. The newly developed multiplex real-time PCR was compared to the previously used single-target PCR protocols for *Chlamydia* spp.,[14] *C. burnetii*,[8] and *N. caninum*.[2] To monitor amplification conditions and to detect PCR inhibitors, an internal control based on the housekeeping gene beta-actin[18] was integrated into the multiplex PCR.

DNA was extracted from the clinical specimen and isolates of bacteria (listed in Table 1) using a commercial DNA extraction kit[a] according to the manufacturer's instructions. The multiplex PCR reaction was carried out in a 25-μl reaction volume. For a single reaction, the PCR mastermix contained 0.8 μl of each primer (10 pmol/μl working solution, final concentration of 320 nM), 0.4 μl of each probe (10 pmol/μl working solution, final concentration of 160 nM), 12.5 μl of PCR buffer containing DNA polymerase,[b] and 0.5 μl of RNase-free water. Finally, 4.0 μl of DNA template was added. The PCR was carried out in a commercial real-time PCR system[c] with an initial denaturation for 15 min at 95°C, followed by 45 cycles of 1 min at 94°C and 90 sec at 60°C.

From the State Institute for Consumer Protection of Saxony-Anhalt, Department of Veterinary Medicine, Stendal, Germany (Reisberg, Gaede), and the Department of Animal Medicine, Faculty of Veterinary Medicine, Benha University, Moshtohor Toukh, Egypt (Selim).

[1]Corresponding Author: Wolfgang Gaede, State Institute for Consumer Protection of Saxony-Anhalt, Department of Veterinary Medicine Haferbreiter Weg 132-135, 39576 Stendal, Germany. wolfgang.gaede@lav.ms.sachsen-anhalt.de

Table 1. Results showing the analytical specificity of the real-time multiplex polymerase chain reaction (PCR) assay by analyzing different targets.*

Target	Matrix/origin	Results of multiplex PCR		
		Chlamydia spp.	*Coxiella*	*Neospora*
Animal species				
Cattle	Ear tissue	No Cq	No Cq	No Cq
Sheep	Blood	No Cq	No Cq	No Cq
Goat	Liver and kidney	No Cq	No Cq	No Cq
Roe deer	Spleen and kidney	No Cq	No Cq	No Cq
Swine	Lung and heart	No Cq	No Cq	No Cq
Viral species				
BDV	BDV Moredun/4. SFT (ref. strain FLI, 2002)	No Cq	No Cq	No Cq
BHV-1	Vaccine Bayovac	No Cq	No Cq	No Cq
BHV-3/MCF	Ref.-DNA (LGL, Germany 2005)	No Cq	No Cq	No Cq
BHV-4	DN 599/8 /1. KOP (ref. strain FLI, 2002)	No Cq	No Cq	No Cq
BRSV	Cs 1432/4 (2006), cattle, nasal swab	No Cq	No Cq	No Cq
BTV	Cs 8808 (2007), cattle, blood	No Cq	No Cq	No Cq
BVDV-1	Cs 533/1 (2008), cattle, blood	No Cq	No Cq	No Cq
BPV-3	UMEA/148KH/15.KOP (ref. strain FLI, 2002)	No Cq	No Cq	No Cq
Bacteria				
Brucella abortus	Bact. suspension (FLI Jena, 2005)	No Cq	No Cq	No Cq
Campylobacter fetus	Cs 1163/2 (2002), cattle	No Cq	No Cq	No Cq
Chlamydia abortus	Cs 26 (2004), sheep, abortion	Positive	No Cq	No Cq
Coxiella burnetii	Cs 1928 (2008) sheep, spleen	No Cq	Positive	No Cq
Escherichia coli	Ref.-DNA, JLU Giessen (1996)	No Cq	No Cq	No Cq
Leptospira	Cs 2689 (2005), swine, kidney	No Cq	No Cq	No Cq
Listeria monocytogenes	Field isolate (1998), sheep	No Cq	No Cq	No Cq
Mycoplasma bovis	Cs 3654/79 (2005), cattle, milk	No Cq	No Cq	No Cq
Mycobacterium avium subsp. paratuberculosis	Cs 3408 (2008), cattle, feces	No Cq	No Cq	No Cq
Neospora caninum	Cs 472/06 (2006), cattle, abortion	No Cq	No Cq	Positive
Pasteurella multocida	Cs 500/1 (2001), cattle, lung	No Cq	No Cq	No Cq

* BDV = *Border disease virus*; BHV-1 = *Bovine herpesvirus 1*; BHV-3 = *Bovine herpesvirus 3*; MCF = malignant catarrhal fever; BHV-4 = *Bovine herpesvirus 4*; BSRV = *Bovine respiratory syncytial virus*; BTV = *Bluetongue virus*; BVD-1 = *Bovine viral diarrhea virus 1*; BPV-3 = *Bovine parainfluenza virus 3*; SFT = sheep fetal thymus; FLI = Friedrich-Loeffler-Institute; LGL = Bavarian Health and Food Safety Authority, Oberschleissheim; KOP = calf oesopharynx; JLU = Justus-Liebig University; Cs = clinical sample; KH = calf testicular cells; Cq = quantification cycle.

For analytical specificity, samples from different animal species and DNA of various viral and bacterial pathogens were examined. The samples showed no signs of nonspecific amplification (Table 1). Analytical sensitivity was determined by using a 10-fold dilution series of target-specific plasmid DNA[d] ranging from 10^9 to 10^0 copies per ml. Dilution series of each plasmid were examined 3-fold; mean values and standard deviation were calculated.[15] For each target, a strong correlation to the respective quantification cycle (Cq) value was found. The reproducible detection limit was 0.13 copies per reaction for each target (Fig. 1). To exclude the possible inhibition of a surplus of competitive DNA, serial dilution ranging from 0.02 to 200 plasmid copies of each target were analyzed with and without a fixed concentration of 10^5 plasmid copies of the respective competing agent. The addition of 10^5 copies of each competing target per reaction did not adversely impact the detection limits for

the specific agent in any case. Therefore, inhibition of amplification due to a high surplus of certain targets can be excluded (Table 2). To compare the sensitivity between the multiplex real-time PCR and respective single-target PCR for *Chlamydia* spp., *N. caninum*, and *C. burnetii*, dilution series of 1 defined clinical reference sample for each target were examined in quadruplicate with multiplex real-time PCR and in triplicate with previous PCR methods. This comparison revealed identical and reproducible detection limits with the tendency for higher sensitivity of the multiplex assay (Table 3).

For diagnostic sensitivity and implementation, 91 samples positive for *Chlamydia* spp., obtained from Saxony-Anhalt (Germany), were analyzed (42 samples/abortion material; 49 samples/nonruminant or nongenital). The nongenital samples in the study included conjunctival and nasal swabs, bronchioalveolar lavage, feces, and tissue homogenates.
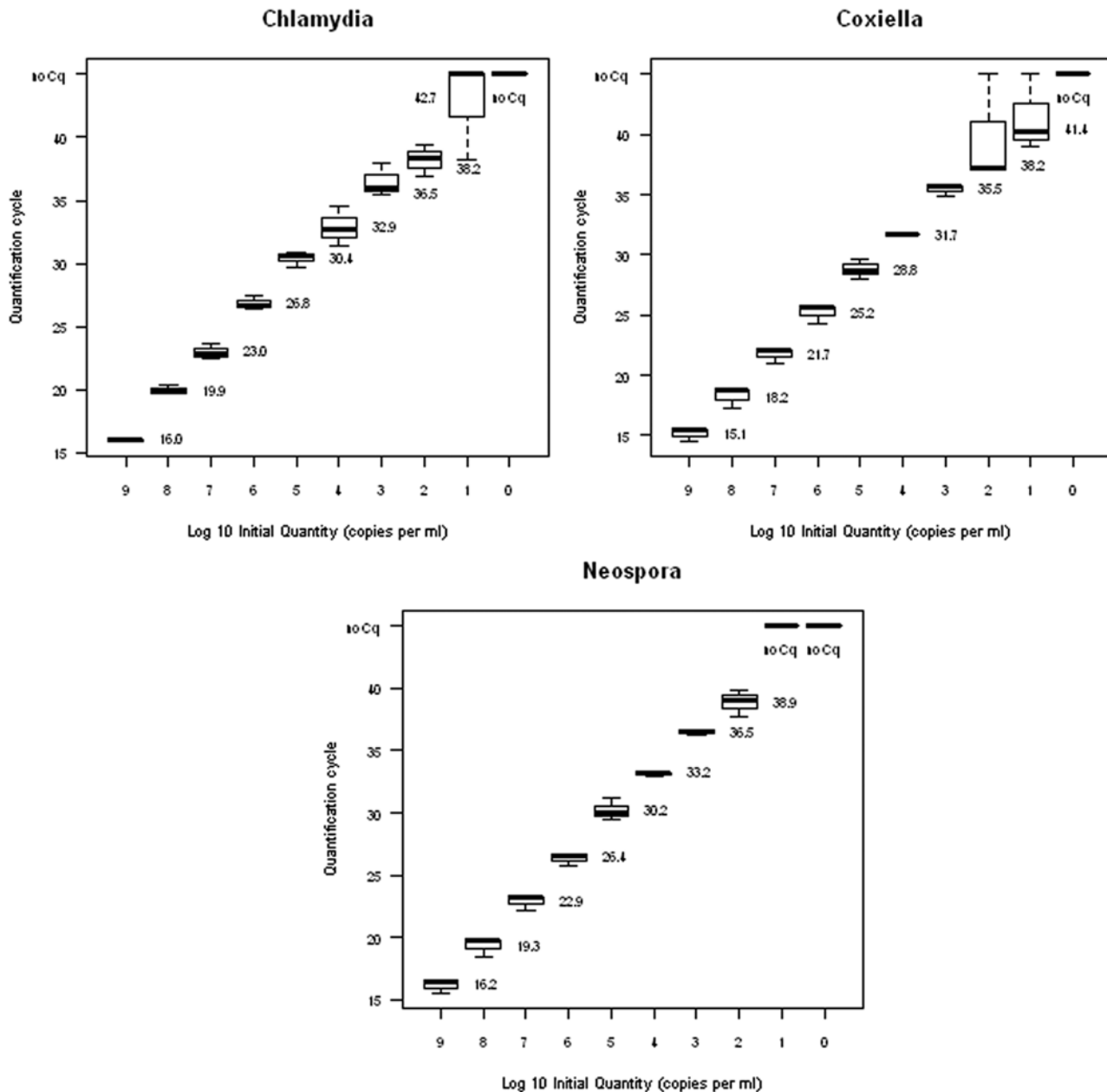
Figure 1. Analytical sensitivity of the assays included in the multiplex real-time polymerase chain reaction determined by dilution series of the respective synthetic plasmids. Mean quantification cycle (Cq) values of the replicates of each dilution step are depicted alongside each box plot. Boxplots were designed supported by the R software package.[15]

Nonruminant samples originated from birds, horses, cats, and swine. Nineteen positive samples for *C. burnetii* were obtained from abortive material from the Bavarian Health and Food Safety Authority (Erlangen, Germany: 3 samples; Oberschleissheim, Germany: 16 samples). Thirty-eight samples positive for *N. caninum* were obtained from the Bavarian Health and Food Safety Authority (Oberschleissheim, Germany: 27 samples) and the Institute of Epidemiology (Friedrich-Loeffler-Institute, Wusterhausen, Germany: 11 samples). For the detection of *Chlamydia* spp., the multiplex

real-time PCR was compared to the results obtained by single-target PCR.[14] Positive results were found with both methods for all 91 clinical samples (Fig. 2). Some of the *Chlamydia* spp.–positive samples have been further identified to species level by species-specific real-time PCR, sequencing, and/or microarray analysis[17] at the Institute of Molecular Pathogenesis of the Friedrich-Loeffler-Institute (Jena, Germany; data not shown). *Chlamydia psittaci*, *Chlamydophila abortus*, *Chlamydophila caviae*, *Chlamydia pecorum*, and *Chlamydophila felis* were identified. Nineteen

Table 2. Analytical sensitivity of multiplex real-time polymerase chain reaction with parallel amplification of 3 targets.

Plasmid copies of specific target per reaction and surplus of possible antagonistic DNA	Quantification cycle (Cq) values for:			
	Chlamydia spp.	*Coxiella*	*Neospora*	Beta-actin
Chlamydia spp.				
200	30.1	No Cq	No Cq	36.5
20	33.9	No Cq	No Cq	37.1
2	36.1	No Cq	No Cq	37.4
0.2	No Cq	No Cq	No Cq	37.0
0.02	No Cq	No Cq	No Cq	38.3
200	29.6	19.4	21.2	37.0
20	33.0	19.4	21.2	36.0
$2 + 10^5$ *Neospora* $+ 10^5$ *Coxiella*	36.7	20.0	20.7	37.1
0.2	37.7	18.3	20.5	36.9
0.02	No Cq	19.3	21.5	36.6
Coxiella burnetii				
200	No Cq	31.9	No Cq	39.0
20	No Cq	33.5	No Cq	35.0
2	No Cq	36.4	No Cq	36.5
0.2	No Cq	No Cq	No Cq	37.0
0.02	No Cq	No Cq	No Cq	36.2
200	21.2	29.0	21.4	35.4
20	21.0	31.6	21.5	35.3
$2 + 10^5$ *Neospora* $+ 10^5$ *Chlamydia* spp.	20.7	34.1	22.3	36.0
0.2	20.7	35.5	22.0	34.8
0.02	20.8	No Cq	21.4	35.2
Neospora caninum				
200	No Cq	No Cq	31.5	37.6
20	No Cq	No Cq	33.1	37.4
2	No Cq	No Cq	37.6	36.7
0.2	No Cq	No Cq	No Cq	37.2
0.02	No Cq	No Cq	No Cq	36.6
200	21.4	21.2	30.0	36.1
20	21.1	21.1	31.5	37.3
$2 + 10^5$ *Coxiella* $+ 10^5$ *Chlamydia* spp.	21.1	21.4	31.4	36.8
0.2	21.5	21.3	33.9	37.6
0.02	21.3	21.6	32.4	36.3

Table 3. Quantification cycle (Cq) values and results for diluted clinical reference samples tested in quadruplicate with the multiplex real-time polymerase chain reaction (PCR) and in triplicate with a previously published PCR method.

	Chlamydia spp.		*Coxiella burnetii*		*Neospora caninum*	
	Clinical sample 83/03		Clinical sample 1/08		Clinical sample 1/09	
Dilution	Multiplex real-time PCR	Single-target real-time PCR	Multiplex real-time PCR	Standard PCR	Multiplex real-time PCR	Nested PCR
Undiluted	30.3	29.8	33.2	Positive	28.8	Positive
5-1	32.5	32.0	36.0	Positive	31.1	Positive
5-2	35.5	35.4	39.0	Negative	34.4	Positive
5-3	37.8*	39.9†	No Cq	Negative	40.6†	Negative
5-4	37.8‡	No Cq	No Cq	Negative	No Cq	Negative
5-5	No Cq	No Cq	No Cq	Negative	No Cq	Negative

* Positive in only 3 replicates.
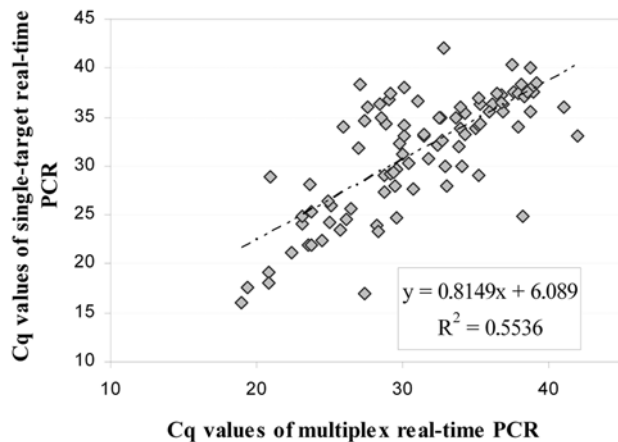† Positive in 2 replicates.
‡ Positive in only 1 replicate.

Figure 2. Correlation between the multiplex and single-target real-time polymerase chain reaction (PCR) detecting *Chlamydia* spp. for 91 positive samples obtained from various host species. Cq = quantification cycle.
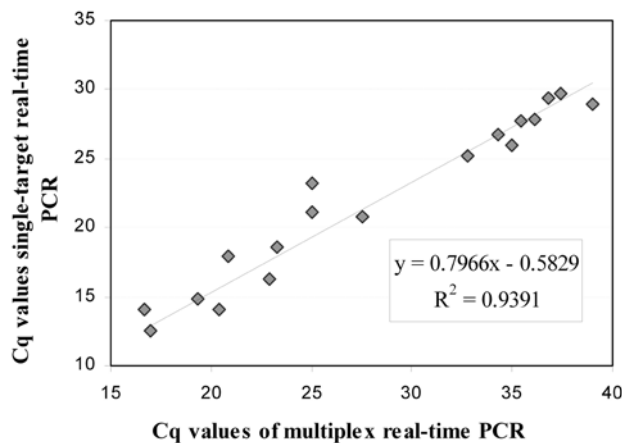

Figure 3. Correlation between the multiplex real-time polymerase chain reaction (PCR) and the single-target real-time PCR targeting the insertion sequence 1111[11] of *Coxiella burnetii* (results obtained by at the Bavarian Health and Food Safety Authority, Erlangen and Oberschleissheim, Germany) for 19 *Coxiella burnetii*–positive samples. Cq = quantification cycle.

C. burnetii–positive samples were analyzed with the multiplex real-time PCR in comparison to the single-target PCR.[8] All positive results were confirmed by the current multiplex real-time PCR (Fig. 3). Although the mean Cq value of the multiplex real-time PCR is increased by 6, the results of both assays are in strong linear correlation. All 38 analyzed *N. caninum*–positive field samples were confirmed by both multiplex real-time PCR and the previously used conventional semi-nested PCR.[2]

The current study shows that the multiplex real-time PCR is able to simultaneously detect *Chlamydia* spp., *C. burnetii*, and *N. caninum* in abortive material of cattle and small ruminants as well as *Chlamydia* spp. in different sample types of ruminant and nonruminant species. Because of the diversity

of *Chlamydia* spp. present in ruminants, the multiplex PCR was deliberately limited to screening for *Chlamydia* at the level of the genus.[4,14] After positive detection of *Chlamydia* spp. DNA in clinical samples, further genotyping can be achieved by the application of species-specific real-time PCR or microarrays.[17] The application of only 1 fluorescence channel for *Chlamydia* spp. allows simultaneous detection of *N. caninum* as an additional, and important, pathogen, in contrast to conventional multiplex PCR assays previously described.[3]

One common problem of multiplex PCR assays is the possible interaction of different oligonucleotides in the same reaction tube, leading to possible inhibition of the amplification reaction and subsequent reduced sensitivity of 1 or even all involved reactions.[9] In the current multiplex PCR, the sensitivity was not found to be influenced by the co-amplification of the 2 additional targets and achieved at least the same sensitivity as the respective single-target PCR when analyzing clinical samples. In conclusion, the current multiplex real-time PCR is an appropriate tool for the detection of *Chlamydia* spp., *C. burnetii*, and *N. caninum* in ruminants in a single reaction and may reduce the expenditures for biochemical reagents, equipment, and labor, resulting in a fast diagnosis for potential zoonotic infections.

Sources and manufacturers

a. High Pure PCR Template Preparation Kit, Roche Diagnostics GmbH, Mannheim, Germany.
b. QuantiTect Multiplex PCR NoRox Kit, Qiagen GmbH, Hilden, Germany.
c. Mx3005, Agilent Technologies, Santa Clara, CA.
d. Gene Express, Berlin, Germany.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

References

1. Aitken ID: 1989, Clinical aspects and prevention of Q fever in animals. Eur J Epidemiol 5:420–424.

2. Baszler TV, Gay LJ, Long MT, Mathison BA: 1999, Detection by PCR of *Neospora caninum* in fetal tissues from spontaneous bovine abortions. J Clin Microbiol 37:4059–4064.

3. Berri M, Rekiki A, Boumedine KS, Rodolakis A: 2009, Simultaneous differential detection of *Chlamydophila abortus, Chlamydophila pecorum* and *Coxiella burnetii* from aborted ruminant's clinical samples using multiplex PCR. BMC Microbiol 9:130.

4. Blumer S, Greub G, Waldvogel A, et al.: 2011, *Waddlia, Parachlamydia* and *Chlamydiaceae* in bovine abortion. Vet Microbiol 152:385–393.

5. Constantin EM, Schares G, Grossmann E, et al.: 2011, Untersuchungen zur Rolle des Rotfuchses (*Vulpes vulpes*) als möglicher Endwirt von *Neospora caninum* [Studies on the role of the red fox (*Vulpes vulpes*) as a potential definitive host of *Neospora caninum*]. Berl Munch Tierarztl Wochenschr 124:148–153. In German. Abstract in English.

6. Delsing CE, Kullberg BJ, Bleeker-Rovers CP: 2010, Q fever in the Netherlands from 2007 to 2010. Neth J Med 68:382–387.

7. Dubey JP, Schares G, Ortega-Mora LM: 2007, Epidemiology and control of neosporosis and *Neospora caninum*. Clin Microbiol Rev 20:323–367.

8. Edingloh M, Merck CC, Manz E: 1999, Multiplex-PCR zum diagnostischen Nachweis von *Coxiella burnetii* aus Kuhmilch [Multiplex PCR for the diagnostic detection of *Coxiella burnetii* in cow's milk]. Berl Munch Tierarztl Wochenschr 112: 5–9. Article in German. Abstract in English.

9. Elnifro EM, Ashshi AM, Cooper RJ, Klapper PE: 2000, Multiplex PCR: optimization and application in diagnostic virology. Clin Microbiol Rev 13:559–570.

10. Hyde SR, Benirschke K: 1997, Gestational psittacosis: case report and literature review. Mod Pathol 10:602–607.

11. Klee SR, Tyczka J, Ellerbrok H, et al.: 2006, Highly sensitive real-time PCR for specific detection and quantification of *Coxiella burnetii*. BMC Microbiol 6:2.

12. Masala G, Porcu R, Daga C, et al.: 2007, Detection of pathogens in ovine and caprine abortion samples from Sardinia, Italy, by PCR. J Vet Diagn Invest 19:96–98.

13. Nietfeld JC: 2001, Chlamydial infections in small ruminants. Vet Clin North Am Food Anim Pract 17: 301–314, vi.

14. Pantchev A, Sting R, Bauerfeind R, et al.: 2010, Detection of all *Chlamydophila* and *Chlamydia* spp. of veterinary interest using species-specific real-time PCR assays. Comp Immunol Microbiol Infect Dis 33:473–484.

15. R Development Core Team: 2009, R: a language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria.

16. Reichel MP, Alejandra Ayanegui-Alcérreca M, Gondim LF, Ellis JT: 2013, What is the global economic impact of *Neospora caninum* in cattle—the billion dollar question. Int J Parasitol 43:133–142.

17. Sachse K, Hotzel H, Slickers P, et al.: 2005, DNA microarray-based detection and identification of *Chlamydia* and *Chlamydophila* spp. Mol Cell Probes 19:41–50.

18. Wernike K, Hoffmann B, Kalthoff D, et al.: 2011, Development and validation of a triplex real-time PCR assay for the rapid detection and differentiation of wild-type and glycoprotein E-deleted vaccine strains of *Bovine herpesvirus type 1*. J Virol Methods 174:77–84.

19. Weston JF, Heuer C, Parkinson TJ, Williamson NB: 2012, Causes of abortion on New Zealand dairy farms with a history of abortion associated with *Neospora caninum*. N Z Vet J 60:27–34.