Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 67 (2007) 303–310
Theriogenology
www.theriojournal.com

Prevalence of chlamydiae in semen and genital tracts of bulls, rams and bucks

K. Teankum [a,e], A. Pospischil [a], F. Janett [b], E. Brugnera [a], L.E. Hoelzle [c], K. Hoelzle [c], R. Weilenmann [a], D.R. Zimmermann [d], A. Gerber [a], A. Polkinghorne [a], N. Borel [a,*]

[a] *Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zürich, Winterthurerstrasse 268, CH-8057 Zürich, Switzerland*
[b] *Clinic of Reproduction, Vetsuisse Faculty, University of Zürich, Winterthurerstrasse 268, CH-8057 Zürich, Switzerland*
[c] *Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Zürich, Winterthurerstrasse 268, CH-8057 Zürich, Switzerland*
[d] *Institute of Clinical Pathology, University Hospital, Schmelzbergstrasse 12, CH-8091 Zürich, Switzerland*
[e] *Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Henri Dunant Road, Pathumwan, 10330 Bangkok, Thailand*

Received 28 April 2006; received in revised form 29 June 2006; accepted 25 July 2006

Abstract

Chlamydiae infect male genital organs of ruminants. However, little is known about their prevalence. Hence, we investigated fresh and cryopreserved semen (bulls: $n = 304$; rams: $n = 78$; bucks: $n = 44$) by polymerase chain reaction (PCR), as well as genital organs (bulls: $n = 13$; rams: $n = 10$; bucks: $n = 6$) by immunohistochemistry (IHC) and PCR. Sera from bulls ($n = 104$) and small ruminants ($n = 61$) were tested by LPS and rMOMP (recombinant major outer membrane protein) ELISA and competitive ELISA (cELISA), respectively. Three PCR assays were compared in this study for detection of chlamydial DNA in semen: 16S rRNA, IGS-S (intergenic spacer 16S/23S-short), and IGS-L (intergenic spacer 16S/23S-long) PCRs. PCR sensitivity and inhibitory effects were determined by spiking semen with *Chlamydophila* (*Cp.*) *abortus* DNA. In bull semen, detection limits of the 16S, IGS-S and IGS-L PCRs were 10, 10, 100 templates, respectively. However, PCR sensitivity was reduced in ram and buck semen suggesting the presence of potential PCR inhibitors. Of 304 bull semen samples, the 16S PCR revealed DNA of chlamydiae in 20 samples (6.6%), including *Cp. abortus* ($n = 2$), *Cp. psittaci* ($n = 1$), *Chlamydia suis* ($n = 2$), and *Chlamydia*-like organisms ($n = 15$). In rams, one semen sample was positive for *Chlamydia*-like organism. All investigated male genital organs were negative for *Chlamydia*. Serology revealed 47.1% (49/104) positive bulls by LPS ELISA. Of these, 30 samples were positive by rMOMP ELISA, predominantly for *Cp. pecorum*. In small ruminants, cELISA displayed 34.8% (16/46) and 60% (9/15) positivity for *Cp. abortus* in rams and bucks, respectively. There was no correlation between serology and PCR of semen. The presence of chlamydiae in semen suggests the possibility of venereal transmission, although risk may be low in Switzerland.

Keywords: Ruminants; Semen; *Chlamydia*; PCR; Prevalence

1. Introduction

Chlamydial infections in ruminants cause a wide range of diseases including polyarthritis, conjunctivitis, pneumonia and abortion [1–4]. According to the reclassification [5], chlamydiae responsible for the diseases in ruminants are *Chlamydophila* (*Cp.*) *abortus*, *Cp. pecorum* (members of the family Chlamydiaceae), and *Waddlia chondrophila* (member of the family Waddliaceae). Of these, *Cp. abortus* is the leading infectious cause of abortion in sheep and goats [6,7]. However, abortion in cattle due to this species is less

* Corresponding author. Tel.: +41 44 635 8576; fax: +41 44 635 8934.
E-mail address: n.borel@access.unizh.ch (N. Borel).

0093-691X/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2006.07.012

common [8,9]. *Cp. pecorum* also causes several diseases in ruminants such as enteritis, polyarthritis, and endometritis [4,8]. Recently, the novel species *Waddlia chondrophila* has been reported from cases of bovine abortion [10,11].

Natural infections of chlamydiae have been reported in bulls and rams since the 1960s [12,13]. Subsequently, *Chlamydia* was isolated from the testes, epididymides and semen of bulls with seminal vesiculitis [13]. The organism was also recognised as a cause of epididymitis in rams [14]. Organisms from experimental infections were successfully re-isolated from genital organs, confirming the capability of the organism to cause an infection of the male genital tract [14,15]. These observations are of great importance particularly for bovine industry as artificial insemination (AI) is widely used and especially since it was shown that *Chlamydia* can survive in cryopreserved semen [13]. The evidence of chlamydial infections in male genital tracts of ruminants indicates the possibility of venereal transmission. The significance of this event under natural conditions is, however, controversial and information concerning prevalence of chlamydial infection in male ruminants is scarce. The aim of the current study was to investigate the prevalence of chlamydial infection in semen and male genital tracts of bulls, rams, and bucks.

Several studies have developed and evaluated PCR techniques to investigate the presence of chlamydial DNA in various clinical samples including semen samples [16–18]. Serological methods, in addition, have also been used to investigate the prevalence of chlamydial infection in ruminants [19–21]. In the current study, we used both serological techniques and several different PCR methods to investigate the prevalence of chlamydial infection in male ruminants.

2. Materials and methods

2.1. Specimens: semen, sera, and male genital organs

Fresh semen and blood samples were collected from three animal species: bulls ($n = 104$, case no. 1-104) aged between 8 months and 7 years, rams ($n = 46$) aged between 8 months and 5 years, and bucks ($n = 15$) aged between 7 and 32 months. All animals were clinically sound, and used in natural breeding, and the semen samples were collected from farms in different regions of Switzerland. All fresh semen samples were aliquotted, and stored at −20 °C prior to testing. Blood samples were centrifuged (3000 × *g*, 10 min) and sera were stored at −20 °C prior to testing.

Cryopreserved semen (bulls: $n = 200$, case no. 105-304; rams: $n = 32$; bucks: $n = 29$) was obtained from different AI centres. Cryopreserved bull semen samples were derived from four AI centres (AI-1: $n = 23$; AI-2: $n = 34$; AI-3: $n = 19$; AI-4: $n = 124$), and cryopreserved semen of small ruminants were obtained from three AI centres (AI-3: $n = 15$; AI-4: $n = 7$; AI-5: $n = 39$).

Male genital organs (bulls: $n = 13$; rams: $n = 10$; bucks: $n = 6$), aged between 4 months and 4 years, were collected from necropsy submissions at the Veterinary Pathology Institute, University of Zurich. Most animals were free from reproductive problems, except a ram with evidence of orchitis, a ram with epididymitis, two bulls suspected for epididymitis, and two bulls with evidence of hypogonadism. Three sections (proximal, middle, and distal parts) were collected from each side of the testis. Other parts of the genital organs including three sections of the epididymides (head, body, and tail), ampulla, seminal vesicles, bulbourethral glands, and four parts of the urethra (proximal, sigmoid flexure, distal and glans penis) were sampled. All organ samples were fixed in 4% formalin, embedded in paraffin and further processed for immunohistochemistry.

2.2. DNA extraction

Extraction of DNA from semen samples was performed using a commercial DNA extraction kit (DNeasy™ Tissue kit, Qiagen, Hilden, Germany), according to the body fluid protocol, using 200 μl of bull semen and 50 μl of semen samples from rams and bucks. DNA was finally eluted in 200 μl of elution buffer (Qiagen). For genital organs, DNA was extracted from paraffin blocks using the protocol as previously described [22].

2.3. Polymerase chain reaction (PCR) and DNA sequencing

To detect chlamydial DNA in semen samples, three PCR assays were evaluated:

(i) *16S rRNA PCR*: The primer pair 16S-IGF (5′-GATGAGGCATGCAAGTCGAACG-3′) and 16S-IGR (5′-CCAGTGTTGGCGGTCAATCTCTC-3′) [5] was designed to amplify 278-bp product of the 16S rRNA gene specific for the order Chlamydiales.

(ii) *IGS-short (IGS-S) PCR*: The primer pair cIGS-1f (5′-CAAGGTGAGGCTGATGAC-3′) and cIGS-2r (5′-TCGCCTKTCAATGCCAAG-3′) was designed to target a variable region of chlamydial DNA

approximately 370-bp PCR product including 80-bp of the 16S rRNA gene, 240-bp of the rRNA intergenic spacer (IGS) region (depending on chlamydial strain), and 50-bp of the 23S rRNA gene.

(iii) *IGS-long (IGS-L) PCR*: The primer pair cIGS-1f (5′-CAAGGTGAGGCTGATGAC-3′) and IGS-1r (5′-AGTGGTCTCCCCAGATTC-3′) were designed to amplify approximately 750-bp PCR product containing 80-bp of the 16S rRNA gene, 240-bp of the IGS region (of variable length between chlamydial species), and 440-bp of the 23S rRNA gene.

The conditions of 16S, IGS-S and IGS-L PCRs are described elsewhere [9,22,23]. Negative and positive control for PCR reactions were performed as previously described [22]. All PCR reactions were carried out in a TGRADIENT thermal cycler (Biometra, Göttingen, Germany). PCR products (5 μl) from all PCR assays were separated by electrophoresis in 1.5% agarose gels and visualised with a UV transilluminator. PCR products were purified by a DNA purification kit (Qiagen), and directly sequenced at the sequencing service of the University of Zurich. The obtained sequences were compared with sequences available in GenBank using the BLAST server from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/).

2.4. Sensitivity test of PCR

To assess the sensitivity of PCR assays, semen samples were spiked with a known amount of *Cp. abortus* S26/3 genomic DNA. The genomic DNA of *Cp. abortus*, prepared as previously described [9,22] was 10-fold diluted in dH_2O, and the diluted DNA was used in spiking experiments as follows:

(i) Bull semen samples (200-μl aliquots of selected fresh and cryopreserved semen) were spiked with a known amount of *Cp. abortus* DNA to obtain the final concentration of 10^3, 10^2, 10, and 1 template/μl of semen samples, respectively.

(ii) Ram semen samples (40-μl aliquots of selected fresh and cryopreserved semen) were spiked with a known amount of *Cp. abortus* DNA to obtain the final concentration of 10^3, 10^2, 10, and 1 template/μl of semen samples, respectively.

(iii) Buck semen samples (40-μl aliquots of selected fresh and cryopreserved semen) were spiked with a known amount of *Cp. abortus* DNA to obtain the final concentration as in Experiment (ii).

DNA was extracted from all spiked semen samples using a commercial DNA extraction kit (DNeasy™ Tissue kit, Qiagen), according to the body fluid protocol, and was finally eluted in 200 μl of elution buffer (Qiagen). The DNA extraction products from all dilution series were compared between all three PCR assays: 16S, IGS-S, and IGS-L PCRs.

2.5. Immunohistochemistry (IHC)

Paraffin sections from all parts of the genital organs were cut and labelled for the presence of chlamydial antigen using a Chlamydiaceae family-specific mouse monoclonal antibody (Ab) directed against the chlamydial lipopolysaccharide (cLPS; clone AC-1, Progen, Heidelberg, Germany) and the streptavidin peroxidase method (DAKO, ChemMate™, Glostrup, Denmark) as previously described [9,22].

2.6. Serological tests

2.6.1. Lipopolysaccharide (LPS) ELISA

Bulls' sera ($n = 104$) were examined by LPS-ELISA. The method was performed as previously described [24]. Briefly, microtiter plates were coated with 40 ng per well of antigen (LPS prepared from EBs of *Cp. abortus* strain OCLH196). Incubations with serum dilutions (1:100) and horseradish peroxidase-conjugated goat anti-bovine IgG (H + L chain specific, Sigma, Buchs, Switzerland) were performed for 1 h. Antigen-antibody reactions were visualised with ABTS (Roche, Rotkreuz, Switzerland) according to the manufacturer's recommendations. Optical densities (OD) were measured at 405 nm by a computer-assisted microplate reader (Tecan, Maennedorf, Switzerland). Cut-off values were calculated for each microtiter plate from mean OD values of seven negative serum samples according to the method of Tijssen [25].

2.6.2. Recombinant major outer membrane protein (rMOMP) ELISA

All LPS-ELISA-positive bull sera ($n = 49$) were examined by rMOMP ELISA using purified recombinant major outer membrane protein (rMOMP) of *Cp. abortus, Cp. pecorum*, and *Cp. psittaci* as antigens as described elsewhere [26,27]. All sera were diluted 1:100 and pre-adsorbed with *Escherichia* (*E.*) *coli* cells overnight at 4 °C to remove antibodies against *E. coli* residues in the rMOMP antigens. To control the pre-adsorption, all sera were tested against purified *E. coli*. OD values were calculated as netto OD values ($OD_{rMOMP} - OD_{E.\ coli}$).

2.6.3. Competitive ELISA (cELISA)

Sera of rams ($n = 46$) and bucks ($n = 15$) were investigated by the competitive enzyme-linked immunosorbent assay (cELISA) using the monoclonal antibody mAb 188 directed against the variable segment 1 of the major outer membrane protein of *Cp. abortus* according to the protocol of Salti-Montesanto et al. [28]. The results of the cELISA were expressed as 'percentage of inhibition' corresponding to the antibody concentration in the serum. Inhibition values above 55% were considered positive for infection with *Cp. abortus* (positive cut-off) whereas inhibition values between 30 and 55% were considered questionable, attributable to either *Cp. abortus* or *Cp. pecorum.* Inhibition values below 30% were considered negative [21,28,29].

2.7. Bacterial culture of non-Chlamydia bacteria

An aliquot of fresh semen samples from all animals and one piece of each genital organ were submitted for routine bacteriological examination.

3. Results

3.1. Sensitivity of PCR

The results of sensitivity tests are summarised in Table 1. In fresh and cryopreserved bull semen spiked with *Cp. abortus* DNA, we detected approximately 100, 10 and 10 chlamydial DNA templates/μl of semen samples by the IGS-L, IGS-S, and 16S PCRs, respectively. In fresh and cryopreserved ram semen, we detected approximately 5000, 500 and 500 chlamydial DNA - templates/μl by the IGS-L, IGS-S, and 16S PCRs, respectively. In fresh buck semen, the detection limits of the IGS-L, IGS-S, and 16S PCRs in semen were 500, 50, 50 templates/μl of semen, respectively. Based on the results of these PCR sensitivity tests, the 16S and IGS-S PCRs were selected to test semen samples, and organ samples were examined by the IGS-S PCR method only.

3.2. PCR detection of chlamydiae in semen samples

Of 104 fresh semen samples from bulls, the 16S PCR detected DNA from a variety of chlamydial species. These included: *Cp. abortus* in one sample (1/104, 0.96%), sharing 100% identity to *Cp. abortus* type sequences; *Cp. psittaci* in one sample (1/104, 0.96%) shared 96% identity to available *Cp. psittaci* 16S sequences and; two samples (2/104, 1.9%) shared 98% identity to the 16S rRNA sequence of *Chlamydia* (*C.*) *suis*. DNA from diverse uncultured *Chlamydia*-like organisms in the order Chlamydiales was detected in eight samples (8/104, 7.7%), shared a range of similarities to previously identified *Chlamydia*-like 16S rRNA sequences (89–100%). Comparison of the *Chlamydia*-positive semen and serological findings of these particular animals is shown in Table 2.

Of 200 cryopreserved semen samples from bulls, the 16S PCR revealed DNA of *Cp. abortus* in only one sample (1/200, 0.5%) which was confirmed by DNA sequencing. *Chlamydia*-like organisms sharing a range of sequence similarities (86–100%) to previously described uncultured *Chlamydia*-like organisms were detected in seven samples (7/200, 3.5%, Table 3).

In the semen of small ruminants, the 16S PCR revealed the presence of a *Chlamydia*-like DNA sharing 86% identity to a previously described sequence (GenBank accession no. AY167115.1) in one cryopreserved semen sample from a ram (1/32, 3.1%) originating from AI-5. The remaining ram semen and all semen samples from bucks were negative.

In all semen samples (fresh and cryopreserved) tested in this study, no semen samples were positive by IGS-S PCR.

3.3. PCR and IHC detection of chlamydiae in genital organs

Detection of chlamydiae in the genital organs using IGS-S PCR yielded negative results in all animals. IHC

Table 1
Sensitivity of *Chlamydia*-specific PCRs performed on semen samples of ruminants

Spiking experiments	Detection limits of PCR assays		
	IGS-long (templates/μl)	IGS-short (templates/μl)	16S (templates/μl)
(i) Bull semen (fresh/cryopreserved)[a]	100	10	10
(ii) Ram semen (fresh/cryopreserved)[a]	5000	500	500
(iii) Buck semen (fresh)[a]	500	500	50
(iii) Buck semen (cryopreserved)[a]	500	50	5

[a] Spiked with *Chlamydophila abortus* DNA followed by DNA extraction using kits (Qiagen).

Table 2
Summary of 16S PCR results of fresh semen from bulls ($n = 104$) used in natural breeding and comparison with serological results (LPS/rMOMP ELISA)

Case no.	Age (yrs)	16S PCR semen	GenBank accession no.	Identity (%)	ELISA	
					LPS	rMOMP
25	5	*Cp. abortus*	CR848038.1	100	–	n.d.
29	2.5	*C. suis*	CTU73110	98	–	n.d.
30	1.5	*Cp. psittaci*	AY334531.1	96	–	n.d.
32	1.5	Uncultured chlamydiales	AY013474.1	89	–	n.d.
52	2	Uncultured chlamydiales	AY013443.1	90	+	*Cp. abortus*, *Cp. pecorum*
59	1.5	*C. suis*	CTU73110	98	–	n.d.
60	2.5	Uncultured chlamydiales	AY013453.1	89	–	n.d.
64	1.5	Uncultured chlamydiales	AY013459.1	95	–	n.d.
70	1.5	Uncultured chlamydiales	AY013464.1	90	+	*Cp. pecorum*
79	7.2	Uncultured chlamydiales	AY013472.1	99	+	*Cp. pecorum*
103	1	Uncultured chlamydiales	AY013468.1	97	–	n.d.
104	0.7	*Candidatus rhabdochlamydia*	AY223862.1	100	+	–

n.d.: not done; −: negative; +: positive; yrs: years.

for chlamydial antigen of organ samples was negative in all cases.

3.4. Serological results of bulls (LPS and rMOMP ELISA)

Antibodies against the LPS antigen of chlamydiae were found in 49 (47.1%) of 104 bulls. The LPS-positive bulls were further examined by rMOMP ELISA and we detected antibody against chlamydial species in 39 cases as shown in Table 4.

3.5. Serological results of small ruminants (cELISA)

Antibodies against *Cp. abortus* were detected in 16 (34.8%) of 46 rams and in 9 (60%) of 15 bucks (Table 5). Semen samples from these animals were negative by the 16S and IGS-S PCRs.

3.6. Bacteriology of semen and organ samples

Bacteriological examination of fresh bull semen ($n = 104$) revealed *Arcanobacterium pyogenes* in four samples, *Haemophilus somnus* in two samples, *Proteus mirabilis* in three samples, and *Corynebacterium* spp. in two samples. In fresh ram semen ($n = 46$), bacteriology revealed *Arcanobacterium pyogenes* in four samples, *Escherichia coli* in one sample, and *Pasteurella* spp. in one sample. In fresh buck semen ($n = 15$), we found *E. coli* in one samples, *Staphylococcus* spp. in one sample, and mixed culture of *Staphylococcus* spp. with alpha-hemolytic *Streptococcus* spp. in one sample.

Bacteriological examination of the organ samples revealed *Staphylococcus aureus* from orchitis in a ram, and *E. coli* from a lesion of the epididymitis in a ram. The remaining organ samples from all other animals were negative by bacteriological examination. Two bulls suspected for epididymitis were diagnosed as

Table 3
Summary of 16S PCR results from cryopreserved semen samples (bulls: $n = 200$) originating from different AI centres

Case no.	16S PCR semen	GenBank accession No.	Identity (%)	AI centres
135	Uncultured chlamydiales	AY013472.1	100	AI-3
136	Uncultured chlamydiales	AY013462.1	86	AI-3
197	Uncultured chlamydiales	AY167118.1		AI-3
205	*Cp. abortus*	CR848038.1	100	AI-3
237	Uncultured chlamydiales	AF364577.1	88	AI-3
264	Uncultured chlamydiales	AF097197.1	99	AI-2
278	Uncultured chlamydiales	AY013457.1	94	AI-2
291	Uncultured chlamydiales	AY013398.1	97	AI-1

AI: artificial insemination.

Table 4
Serological results of bulls ($n = 104$) examined by LPS and rMOMP ELISA

No. of LPS (+) bulls	49
No. of bulls positive for rMOMP ELISA ($n = 39$[a])	
Single infection	
Cp. abortus	6
Cp. pecorum	26
Mixed infections	
Cp. abortus/*Cp. pecorum*	5
Cp. abortus/*Cp. psittaci*	1
Cp. abortus/*Cp. pecorum*/*Cp. psittaci*	1

+: positive.
[a] Only LPS positive bulls were tested by MOMP ELISA.

spermatocele and were negative for bacteriology. In other two bulls, hypogonadism was confirmed as clinically expected. The prevalence of bacterial orchitis or epididymitis in all animals examined was 6.9% (2/29 cases).

4. Discussion

In this report, we evaluated three PCR assays (IGS-long, IGS-short, and 16S PCRs) for use in investigating the prevalence of chlamydiae in semen samples from ruminants. Spiking experiments (except Experiment (iii)), designed to assess PCR sensitivity and the presence of potential inhibitors in semen revealed that the sensitivity of the IGS-L PCR was lower than 16S and IGS-S PCRs by at least 10-fold. In semen of small ruminants, sensitivity of all PCR methods used was reduced compared to semen from bulls (in this study) and boars [22] indicating the presence of potential inhibitory factors. The high concentration of spermatozoa in ram and buck semen may contribute to the inhibition of PCR that we observed.

Comparison of fresh and cryopreserved semen from bulls and rams revealed identical detection limits suggesting different preservation methods do not affect PCR detection. In bucks, however, PCR sensitivity was higher in cryopreserved semen compared to fresh semen. As described in another recent study [9], we observed that the 16S was more sensitive than IGS-S PCR (as in Experiment (iii)) for detection of chlamydial DNA in semen samples.

Despite the latter result, both 16S and IGS-S PCRs were used to test semen samples from all animals. Consistently with our recent investigations into the prevalence of *Chlamydia* in semen from boars [22], the results from our 16S rRNA PCR screening revealed a rather low prevalence (6.6%, 20/304) of chlamydial DNA in semen from bulls. It is also worth noting that IGS-S PCR yielded only negative results from these semen samples suggesting that chlamydial DNA is only present in very few copies (i.e. 16S rRNA PCR sensitivity > IGS-S PCR). The results of the current study are contrary to another study reporting a high proportion of bulls positive for *Cp. psittaci* in semen [16], however, their study was limited to analysis of three different herds. Such discrepancy could be due to different sources of animals with different management conditions.

DNA from a number of different chlamydial species including *Cp. abortus* ($n = 2$), *Cp. psittaci* ($n = 1$), *C. suis* ($n = 2$), and *Chlamydia*-like organisms ($n = 15$) were detected by the 16S PCR. *Cp. abortus* is known to be the main infectious cause of abortion in small ruminants, but to a lesser extent in cattle [9]. In this study, DNA of *Cp. abortus* was detected in one fresh semen sample from an asymptomatic bull used in natural breeding and one cryopreserved semen sample of a bull from AI station. Although the prevalence of *Cp. abortus* is very low in this study, its presence in semen samples from bulls indicates the feasibility of venereal transmission. Such cases as they occur would be highly significant since a single *Chlamydia*-positive bull could spread the organism widely via AI.

In contrast to *Cp. abortus*, the significance of *Cp. psittaci* in bovine reproductive diseases is still controversial. However, *Cp. psittaci* was also found to be associated with bovine abortion [9] and the presence of *Cp. psittaci* in bull semen in this study suggests an involvement of this chlamydial species in reproductive diseases. Interestingly, we detected DNA of chlamydiae sharing similarity to *C. suis* in semen of two bulls. The significance of this finding is difficult to interpret since this is the first report of this kind.

Serological findings of these *Cp. abortus*-, *Cp. psittaci*-, and *C. suis*-positive bulls displayed negative

Table 5
Summary of serological results (cELISA) for *Cp. abortus* infection in rams and bucks

Animals (n)	No. of positive animals (%)	No. of negative animals (%)	No. of questionable animals (%)
Rams (46)	16 (34.8)	15 (32.6)	15 (32.6)
Bucks (15)	9 (60)	2 (13.3)	4 (26.6)

results by LPS ELISA. This may be due to the local infection of male genital tracts and semen not inducing systemic immune responses. Data retrieved from the records of breading soundness examination of these bulls showed that they were clinically sound and had normal semen quality without abortion history in the herds. The fact that these particular bulls yielded positive results only by the 16S PCR may indicate that these bulls harboured only small amount of chlamydiae.

In addition to these positive samples, we identified other semen samples containing *Chlamydia*-like organisms, other than traditional members of the family Chlamydiaceae. Detection of DNA from these novel organisms is consistent with a number of other recent studies screening cervical swab from sows [30] and bovine placenta [9]. However, the significance of *Chlamydia*-like organisms in reproductive diseases of animals requires further study as a causal role in the manifestation of clinical diseases remains unproven.

Of a total of 122 semen samples from small ruminants that were screened via PCR, we could detect DNA from a *Chlamydia*-like organism in only one cryopreserved sample from a ram originating from AI station. As previously discussed, PCR sensitivity tests revealed that potential PCR inhibition factors were present in semen from rams. It is not clear whether this low prevalence reflected the actual disease situation in these animals or whether detection sensitivity was reduced by the latter factors.

Serological investigation of chlamydial infections in bulls and small ruminants revealed contrasting results to the PCR analysis. The overall serological prevalence of chlamydial infection in bulls, accessed by LPS ELISA, was 47.1% (49/104). In comparison to other studies, the seroprevalence in bulls in this study is in the same range reported in cows with reproductive problems, despite the fact that different serological methods were employed [31,32].

Among LPS-ELISA-positive bulls, *Cp. pecorum* was most commonly detected (26/49, 53.1%) than *Cp. abortus* (6/49, 12.2%) as determined by rMOMP ELISA. We were also able to detect mixed infections of different chlamydial species occurred in the same bulls (Table 4). Mixed infections between *Cp. abortus* and *Cp. pecorum* (5/49, 10.2%) were more often detected than mixed infections between *Cp. abortus* and *Cp. psittaci* (1/49, 2%) and mixed infections of *Cp. abortus*, *Cp. psittaci* and *Cp. pecorum* (1/49, 2%). A discrepancy between the results of LPS and rMOMP ELISAs was found in 10 bulls which were positive for LPS but negative for rMOMP ELISA. Most of these cases had low OD values close to the positive cut-off OD value and, therefore false positive results of the LPS ELISA were probably due to cross reaction with other Gram-negative bacteria.

Similar discrepancies between serological and PCR results could also be observed by serological investigation (cELISA) of chlamydial infections in small ruminants in which 34.8% of rams (16/46) and 60% of bucks (9/15) were positive for *Cp. abortus*, indicating a previous exposure to this chlamydial species. In this situation, it is most likely that the chlamydial seroprevalence resulted from infection in other anatomical sites of these animals. A previous survey in Switzerland revealed 19% seroprevalence of *Cp. abortus* infection in sheep with higher prevalence in specific regional areas [21]. In the current study, the seroprevalence of *Cp. abortus* infection in rams was higher than the previous survey, however as has been noted, the number of animals investigated between these studies was different.

To further address the question of disease situation in ruminants, we attempted to investigate the distribution of chlamydiae in the genital organs. Considering the low prevalence found in semen samples, we were not surprised to obtain negative results from all genital organs examined by IHC and IGS-S PCR.

In conclusion, we identified the presence of *Cp. abortus* and *Cp. psittaci* in bull semen, the former of which is a known cause of pregnancy complications and abortion in cows. Although this study revealed a low prevalence of chlamydial DNA in semen samples from asymptomatic bulls, wide dissemination of the organisms can still occur if even a single *Chlamydia*-positive bull is used for AI purposes.

Acknowledgements

We are grateful to Swissgenetics (Zollikofen), Select Star SA (Puplinge), Triple-Genetics-Service AG (Selzach) Switzerland, Dr. Enzo Fuschini (Swissgenetics, Bütschwil) and Dr. Catherine Marguerat (Schweizerischer Ziegenzuchtverband, Bern) for providing us cryopreserved semen of ruminants. We thank also the laboratory staff of the Institute of Veterinary Pathology and the Institute of Veterinary Bacteriology, University of Zürich, as well as Anja Hamburger for her assistance in serological test.

References

[1] Storz J, Shupe JL, Smart RA, Thornley RW. Polyarthritis of calves: experimental induction by a psittacosis agent. Am J Vet Res 1966;27:987–95.

[2] White G. Experimental production of pneumonia in calves by infection with an organism of the psittacosislymphogranuloma group. Vet Rec 1965;77:1124–6.
[3] Cox HU, Hoyt PG, Poston RP, Snider TG, Lemarchand TX, O'Reilly KL. Isolation of an avian serovar of *Chlamydia psittaci* from a case of bovine abortion. J Vet Diagn Invest 1998;10:280–2.
[4] Shewen PE. Chlamydial infection in animals. Can Vet J 1980;21:2–11.
[5] Everett KD, Bush RM, Andersen AA. Emended description of the order *Chlamydiales*, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms. Int J Syst Bacteriol 1999;4:415–40.
[6] Nietfeld JC. Chlamydial infections in small ruminants. Vet Clin N Am Food Anim Pract 2001;17:301–14.
[7] Chanton-Greutmann H, Thoma R, Corboz L, Borel N, Pospischil A. Aborte beim kleinen Wiederkäuer in der Schweiz: Untersuchungen während zwei Ablammperioden (1996–1998) unter besonderer Beachtung des Chlamydienabortes. Schweiz Arch Tierheilkd 2002;144:483–92.
[8] Everett KD. Chlamydia and Chlamydiales: more than meets the eye. Vet Microbiol 2000;75:109–26.
[9] Borel N, Thoma R, Spaeni P, Weilenmann R, Teankum K, Brugnera E, et al. *Chlamydia*-related abortions in cattle from Graubunden, Switzerland. Vet Pathol, in press.
[10] Rurangirwa FR, Dilbeck PM, Crawford TB, McGuire TC, McElwain TF. Analysis of the 16S rRNA gene of micro-organism WSU 86-1044 from an aborted bovine foetus reveals that it is a member of the order Chlamydiales: proposal of Waddliaceae fam. nov., *Waddlia chondrophila* gen. nov., sp. nov. Int J Syst Bacteriol 1999;49:577–81.
[11] Henning K, Schares G, Granzow H, Polster U, Hartmann M, Hotzel H, et al. *Neospora caninum* and *Waddlia chondrophila* strain 2032/99 in a septic stillborn calf. Vet Microbiol 2002;85: 285–92.
[12] Ball L, Young S, Carroll EJ. Seminal vesiculitis syndrome: lesions in genital organs of young bulls. Am J Vet Res 1968; 29:1173–84.
[13] Storz J, Carroll EJ, Ball L, Faulkner LC. Isolation of a psittacosis agent (*Chlamydia*) from semen and epididymis of bulls with seminal vesiculitis syndrome. Am J Vet Res 1968;29:549–55.
[14] Lozano EA. Etiologic significance of bacterial isolates from rams with palpable epididymitis. Am J Vet Res 1986;47:1153–6.
[15] Storz J, Carroll EJ, Stephenson EH, Ball L, Eugster AK. Urogenital infection and seminal excretion after inoculation of bulls and rams with chlamydiae. Am J Vet Res 1976;37:517–20.
[16] Domeika M, Ganusauskas A, Bassiri M, Froman G, Mardh PA. Comparison of polymerase chain reaction, direct immunofluorescence, cell culture and enzyme immunoassay for the detection of *Chlamydia psittaci* in bull semen. Vet Microbiol 1994;42:273–80.
[17] Hartley JC, Kaye S, Stevenson S, Bennett J, Ridgway G. PCR detection and molecular identification of Chlamydiaceae species. J Clin Microbiol 2001;39:3072–9.
[18] Amin AS. Comparison of polymerase chain reaction and cell culture for the detection of *Chlamydophila* species in the semen of bulls, buffalo–bulls, and rams. Vet J 2003;166:86–92.
[19] Kaltenboeck B, Heard D, DeGraves FJ, Schmeer N. Use of synthetic antigens improves detection by enzyme-linked immunosorbent assay of antibodies against abortigenic *Chlamydia psittaci* in ruminants. J Clin Microbiol 1997;35:2293–8.
[20] Buendia AJ, Cuello F, Del Rio L, Gallego MC, Caro MR, Salinas J. Field evaluation of a new commercially available ELISA based on a recombinant antigen for diagnosing *Chlamydophila abortus* (*Chlamydia psittaci* serotype 1) infection. Vet Microbiol 2001;78:229–39.
[21] Borel N, Doherr MG, Vretou E, Psarrou E, Thoma R, Pospischil A. Seroprevalences for ovine enzootic abortion in Switzerland. Prev Vet Med 2004;65:205–16.
[22] Teankum K, Pospischil A, Janett F, Bürgi E, Brugnera E, Hoelzle K, et al. Detection of chlamydiae in boar semen and genital tracts. Vet Microbiol 2006;116:146–57.
[23] Lutz-Wohlgroth L, Becker A, Brugnera E, Huat ZL, Zimmermann D, Grimm F, et al. Chlamydiales in guinea pigs and their potential zoonotic risk. J Vet Med A 2006;53:185–93.
[24] Knitz JC, Hoelzle LE, Affolter P, Hamburger A, Zimmermann K, Heinritzi K, et al. Humorale Immunantwort von Zuchtsauen nach Impfung mit einer stallspezifischen *Chlamydophila abortus*-Vakzine. Dtsch Tierarztl Wochenschr 2003;110:369–74.
[25] Tijssen P. Practice and theory of enzyme immunoassays. In: Burdon RH, van Knippenberg PH, editors. Laboratory techniques in biochemistry and molecular biology. Amsterdam: Elsevier Publishing; 1985. p. 385–421.
[26] Hoelzle LE, Hoelzle K, Wittenbrink MM. Expression of the major outer membrane protein (MOMP) of *Chlamydophila abortus*, *Chlamydophila pecorum*, and *Chlamydia suis* in *Escherichia coli* using an arabinose-inducible plasmid vector. J Vet Med B 2003;50:383–9.
[27] Hoelzle LE, Hoelzle K, Wittenbrink MM. Recombinant major outer membrane protein (MOMP) of *Chlamydophila abortus*, *Chlamydophila pecorum*, and *Chlamydia suis* as antigens to distinguish chlamydial species-specific antibodies in animal sera. Vet Microbiol 2004;103:85–90.
[28] Salti-Montesanto V, Tsoli E, Papavassiliou P, Psarrou E, Markey BK, Jones GE, et al. Diagnosis of ovine enzootic abortion, using a competitive ELISA based on monoclonal antibodies against variable segments 1 and 2 of the major outer membrane protein of *Chlamydia psittaci* serotype 1. Am J Vet Res 1997;58:228–35.
[29] Gut-Zangger P, Vretou E, Psarrou E, Pospischil A, Thoma R. Chlamydienabort beim Schaf: Möglichkeiten der serologischen Diagnostik mit einem kompetitiven ELISA und Einblick in die epidemiologische Situation in der Schweiz. Schweiz Arch Tierheilkd 1999;141:361–6.
[30] Camenisch U, Lu ZH, Vaughan L, Corboz L, Zimmermann DR, Wittenbrink MM, et al. Diagnostic investigation into the role of Chlamydiae in cases of increased rates of return to oestrus in pigs. Vet Rec 2004;155:593–6.
[31] Cavirani S, Cabassi CS, Donofrio G, De Iaco B, Taddei S, Flammini CF. Association between *Chlamydia psittaci* seropositivity and abortion in Italian dairy cows. Prev Vet Med 2001;50: 145–51.
[32] Wehrend A, Failing K, Hauser B, Jager C, Bostedt H. Production, reproductive, and metabolic factors associated with chlamydial seropositivity and reproductive tract antigens in dairy herds with fertility disorders. Theriogenology 2005;63:923–30.