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Comparison of polymerase chain reaction and cell culture for the detection of *Chlamydophila* species in the semen of bulls, buffalo-bulls, and rams

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Abstract

Two hundred and thirty six semen samples were collected from 120 bulls, 60 buffalo-bulls, and 56 rams located on farms of known history of infection with *Chlamydophila* species. All semen samples were examined by polymerase chain reaction (PCR) and cell culture techniques for detection of *Chlamydophila* species. The primers were selected to allow the amplification of all target species in a single reaction by identifying conserved sequences in the omp2 gene. PCR assay detected more positive samples (36) from the semen samples collected from different animal species than were detected by the culture method (21). The results indicated that all culture-positive semen samples (21) from different species were PCR positive. The detection limit of the PCR assay was determined with DNA extracted from fourfold serial dilution of *C. abortus* (B577) and *C. pecorum* (11/88) cultures and found to be 0.25 inclusion-forming units (IFU) per PCR, while the culture method could not detect less than 4 IFU. This is the first report using PCR for the detection of *Chlamydophila* species in buffalo-bulls' semen and the assay provides a simple, sensitive, rapid, and reliable means for the detection and identification of the organism.

Keywords: Chlamydophila; PCR; Semen; Buffalo-bulls; Bulls; Rams

1. Introduction

Chlamydiae are unique intracellular pathogens with a biphasic developmental cycle consisting of metabolically inactive and infectious elementary bodies and metabolically active but non-infectious reticulate bodies (Moulder, 1991). The family Chlamydiaceae has recently been reclassified based on 16S and 23S rRNA gene sequences into two genera *Chlamydia* and *Chlamydophila* and nine species *Chlamydia trachomatis*, *C. muridarum*, and *C. suis*, and *Chlamydophila pneumoniae*, *C. psittaci*, *C. abortus*, *C. felis*, *C. pecorum*, and *C. caviae* (Everett et al., 1999).

Chlamydophila species infection have been reported worldwide in the animal kingdom and may lead to overt clinical diseases, such as enteritis, pneumonia, encephalitis, polyarthritis, conjunctivitis, mastitis, and placental and fetal infection with abortion (Storz, 1971; Lazono, 1986). Infections in rams and bulls can result in male infertility and sterility (Storz, 1971; Eaglesome et al., 1992). *Chlamydophila* species may be excreted in semen of infected males intermittently for three weeks following infection (Storz et al., 1976; Shewen, 1986). Chlamydiae have been isolated from the semen of clinically normal bulls (Bicknell et al., 1986) as well as from semen of bulls with seminal vesiculitis syndrome (Storz et al., 1968). Infertility, retained placenta, repeat breeder, abortion, stillbirths or delivery of premature weak kids, lambs, or calves with low birth weight and milk production drop were observed in females naturally or artificially inseminated by infected semen (Storz et al., 1976; Bowen et al., 1978; Appleyard et al., 1985; Rodolakis & Souriau, 1986; Amin et al., 1999).

Artificial insemination (AI) is widely used in the bovine dairy industry and is increasingly available in other

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species (Watson, 1990). In combination with cryopreserved semen, AI is a powerful tool for facilitating genetic exchange and the maintenance of genetically important remnant populations (Watson, 1990). On the other hand, it increases national and international distribution of semen and the risk of spreading pathogenic microorganisms (including *Chlamydophila* species) among the animal population (Appleyard et al., 1985; Philpott, 1994). Before animal semen can be used for AI, it is essential to screen semen for *Chlamydophila* infection to eliminate the risk of disease transmission.

Chlamydophila species infections have been difficult to diagnose either serologically or by isolation of the organism from infected semen samples. Serological assays of *Chlamydophila* species have not yet been well standardized and the use of these tests for large-scale epidemiological studies has been hampered by poor correlation between serological results and sample detection as well as by inter-laboratory variation (Aitken, 1996; Hartley et al., 2001). In addition, *Chlamydophila* species shares common antigens with some Gram-negative bacteria, so that the serological tests are not wholly specific (Aitken, 1996). Furthermore, under certain conditions, including exposure to gamma interferon or antibiotics, Chlamydiae can become dormant and reside in cells in a non-replicating form that may escape immune detection and persist for long periods of time (Beatty et al., 1994). Firm evidence of the presence of Chlamydiae is required for unequivocal diagnosis because of the preponderance of clinically inapparent and persistent infections (Storz, 1988). Cell culture is considered the "gold standard" for detection of *Chlamydophila* species but it is time consuming since isolation from any sample requires more than one passage. In addition, it is laborious and can give variable results depending on the numbers of viable infectious elementary bodies. Moreover, the organism requires level 3 (P3) contaminant facilities for propagation (Storz, 1988; Hartley et al., 2001). Finally, toxic effect of semen on cell culture may lead to improper diagnosis (Aitken, 1996).

Amplification of Chlamydial DNA by the polymerase chain reaction (PCR) provides an alternative approach for verifying the presence of *Chlamydophila* species in biological samples without resorting to culture. PCR assay involves a variety of formats including conventional (non-nested) and nested as well as touchdown enzyme time release (TETR)-PCR. Although several reports have documented the use of these various assays for detection of *Chlamydophila* species in different samples (Hewison et al., 1991; Kaltenboeck et al., 1992; Herring, 1993; Tong & Sillis, 1993; Messmer et al., 1997; Madico et al., 2000; Hartley et al., 2001), to our knowledge there have been no studies that have compared their performance on semen samples or using buffalo-bull semen. The objective of this study was to detect *Chlamydophila* species in naturally infected semen samples, collected from bulls, buffalo-bulls, and rams, by a PCR amplification assay (Hartley et al., 2001) and to compare this to cell culture.

2. Materials and methods

2.1. Semen samples

Two hundred and thirty six semen samples were collected from 120 bulls, 60 buffalo-bulls, and 56 rams from farms of known history of infection with *Chlamydophila*. Each semen sample was diluted in a Tris-buffered–fructose–glycerol–yolk and the mixture was divided into 200 μl aliquots (Foote, 1970) and stored at −70 °C until used. A further 30 semen samples were collected from *Chlamydophila*-free bulls, buffalo-bulls, and rams to be used as negative control samples. To evaluate the PCR limit of *C. abortus* detection in semen, semen samples were collected from *Chlamydophila* species free-bulls and fourfold dilution series of *C. abortus* (B-577) were added (10 samples per dilution). Cultural and PCR amplification assays were applied for detection of the organism in all samples. To evaluate the PCR limit of *C. pecorum* detection in semen, *C. pecorum* (11/88) was used.

2.2. Sample preparations

The non-sperm cells and seminal fluid fractions were separated from each semen sample as described by Von Beroldingen et al. (1990). Briefly, two volumes of lysis buffer (0.15 M NaCl, 0.75% sodium-*N*-lauroylsarcosine, and 1.5 mg of proteinase K [Boehringer] per ml) were added to each semen sample and the mixture was incubated at 60 °C for 1 h. The pooled fraction of non-sperm cells and seminal fluid was obtained from the supernatant. All prepared samples were stored at −70 °C until used.

2.3. Organisms

Three Egyptian isolates of *C. abortus* and *C. pecorum* (Amin, 1993), together with reference standard strains of *C. abortus* (B-577) and *C. pecorum* (11/88) obtained from the Virology Laboratory, University of Arizona, USA and Animal Reproduction Research Institute, Egypt, were used.

2.4. Isolation of Chlamydophila species in cell culture

Cell culture and isolation of *Chlamydophila* was performed as described previously (Amin, 1993). Briefly, standard culture fluid of each strain (200 μl) or 1 ml of each diluted semen sample was inoculated in triplicate into shell vials containing monolayers of baby hamster

kidney (BHK) cells. Inoculated shell vials were centrifiiged at 800*g* for 1 h at 37 °C. Following aspiration of the inoculum, 1 ml of Iscov's medium (GIBCO) supplemented with 10% fetal calf serum, 50 μg/ml gentamicin, 25 unit/ml nystatin, and 1 μg/ml cycloheximide was added to each culture. After 2–3 days incubation at 37 °C and 5% CO_2, a second passage was performed. The cell contents of a shell vial were fixed with 90% methanol and stained with fluorescein isothiocyanate conjugated chlamydial antibodies (provided by the National Animal Disease Center, Ames, IA, USA) to assess the presence of the inclusion bodies. Organisms from two shell vials were stored at −70 °C until used.

Chlamydophila abortus (B-577) and *C. pecorum* (11/88) were titrated in 96-well microtitre plates containing monolayers of BHK cells. One hundred microlitres of 10-fold dilutions of the cultures were inoculated, incubated, fixed, and stained as described above. The number of inclusion bodies counted under the epifluorescence microscope for each well was multiplied by the dilution factor to calculate the number of inclusion forming units (IFU) per millilitre in the stock cultures (Madico et al., 2000).

2.5. DNA extraction

DNA was extracted from cultures and prepared semen samples as described previously (Walsh et al., 1991). Briefly, 50 μl of each sample was added to 200 μl of a 5% suspension of chelating resin (Chelex 100; Sigma) in Tris–HCl buffer (0.01 M, pH 8.2) and incubated at 56 °C for 30 min. After incubation, each prepared sample was mixed gently and heated at 100 °C for 10 min. After being mixed again, the prepared samples were stored at −70 °C until testing and immediately before testing, they were centrifuged at 11,000*g* for 30 s and 10 μl of the supernatant was used for PCR.

2.6. Oligonucleotide primers

The primers were selected to allow the amplification of all target species in a single reaction by identifying conserved sequences in the omp2 gene. The primer sequences as previously described by Hartley et al. (2001) were synthesized using a DNA synthesizer 392 (Applied Biosystems) and purified chromatographically (NAP-10 Sephadex column, Pharmacia). The sequences of oligonucleotide primers were [family specific PCR primer (sense) 5′ ATG TCC AAA CTC ATC AGA CGA G 3′] and [family specific PCR primer (antisense) 5′ CCT TCT TTA AGA GGT TTT ACC CA 3′].

2.7. DNA amplification and detection of PCR product

PCR conditions were performed in a 50 μl reaction volume containing 10 μl of processed sample, 200 μM each of mix of deoxynucleotide triphosphates (dNTPs) (Pharmacia), 5 pmol of each primer, 5 μL of 10× buffer with 15 mM $MgCl_2$ and 1.25 U of Taq polymerase (Roche Biochemicals) (Hartley et al., 2001). Each PCR mixture was overlaid with 40 μl of paraffin oil (Sigma) and amplified in a DNA thermal cycler (Coy Corporation). Amplification conditions of the primers were 94 °C for 4 min for 1 cycle; 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 1 min for 40 cycles; and a final extension step of 72 °C for 7 min for 1 cycle. PCR products (10 μl) were separated by electrophoresis in 2% agarose gels and were visualized with ethidium bromide (0.5 μg/ml) under ultraviolet transilluminator and photographed (Sambrook et al., 1989). A visible band of appropriate size (603 bp) was considered as a positive reaction. To avoid false-positive PCR results, the precautions for PCR described by Kwok and Higuch (1989) were strictly followed.

2.8. Analysis of discrepant results

Semen samples with discrepant results between the PCR and culture for the detection of *Chlamydophila* species were resolved by an additional nested PCR targeting the omp1 gene (Tong & Sillis, 1993).

2.9. Statistical analysis

The sensitivity and specificity of PCR and culture for detection of *Chlamydophila* species in semen samples were calculated by using as a reference gold standard samples that were detected as positive by at least two methods: PCR, culture, or nested PCR (Fleiss, 1981).

3. Results

3.1. Comparison of PCR and culture results for detection of Chlamydophila species in semen samples

The PCR assay amplified *Chlamydophila* species-DNA from 18, 7, and 11 semen samples from bulls, buffalo-bulls, and rams, respectively (Table 1 and Fig. 1). The culture method detected *Chlamydophila* species from 10, 4, and 7 semen samples from bulls, buffalo-bulls, and rams, respectively (Table 1). The additional nested PCR assay amplified *Chlamydophila* species-DNA from 17, 7, and 10 semen samples from bulls, buffalo-bulls, and rams, respectively (Table 1). The results indicated that all culture-positive semen samples (21) from different species were PCR-positive (Table 1). Out of the 15 PCR-positive, culture-negative samples, 13 were confirmed as true positives with an additional nested PCR targeting the omp1 gene (Tong & Sillis, 1993), while two samples were not confirmed and

Table 1
Comparison of PCR and culture for the detection of *Chlamydophila* species in semen of bulls, buffalo-bulls and rams

Species/total samples	Number of semen samples	Results obtained by:			Sensitivity %[a]		Specificity%[a]	
		PCR	Culture	Nested PCR	PCR	Culture	PCR	Culture
Bulls/120	10	+	+	+				
	7	+	–	+	100	58.5	99.0	100
	1	+	–	–				
	102	–	–	–				
Buffalo–bulls/60	4	+	+	+				
	3	+	–	+	100	57.1	100	100
	0	+	–	–				
	53	–	–	–				
Rams/56	7	+	+	+				
	3	+	–	+	100	70.0	97.8	100
	1	+	–	–				
	45	–	–	–				
Total/236	21	+	+	+				
	13	+	–	+	100	61.8	99.0	100
	2	+	–	–				
	200	–	–	–				

+, Positive; –, negative.
[a] The sensitivity and specificity were calculated by using as a reference gold standard samples that were detected as positive by at least two methods: PCR, culture, or nested PCR.

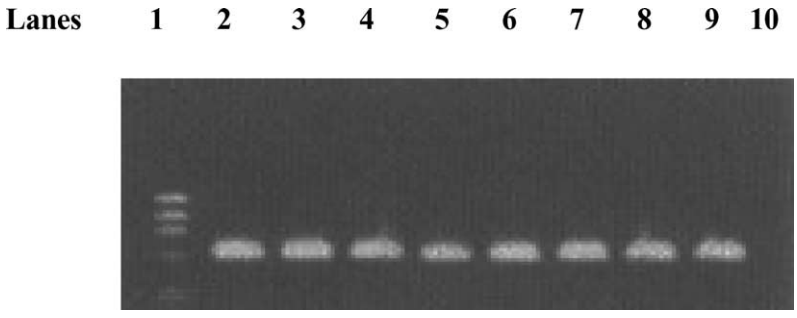

Fig. 1. PCR amplification of *C.* species DNA from semen samples. Lane 1, molecular weight marker; lane 2, bull sample; lane 3, buffalo-bull sample; lane 4, ram sample; lanes 5–7, *C. abortus* isolates; lane 8, *C. pecorum* isolate; lane 9, *C. abortus* B-577 (positive control); and lane 10, negative control.

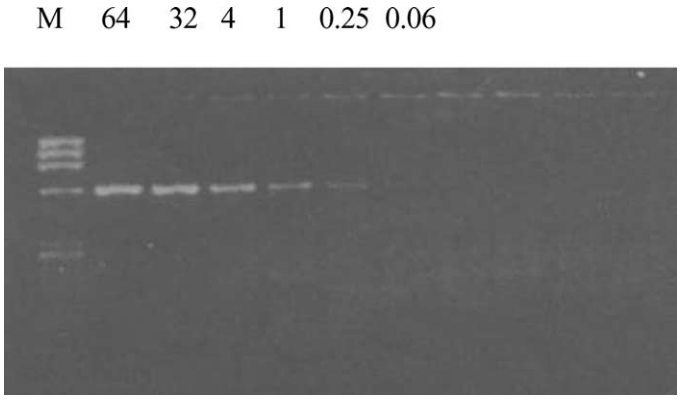

Fig. 2. Detection limits of *C. abortus* (B-577) in semen by PCR. The number of IFU is indicated at the top. Lane M, molecular weight marker.

considered false positives. After discrepant samples were resolved, 17, 7, and 10 of the samples from bulls, buffalo-bulls, and rams, respectively, were judged true positives (Table 1). After confirmation, the PCR sensitivities were 100%, 100%, and 100% while the culture sensitivities were 58.5%, 57.1%, and 70.0% in the semen samples from bulls, buffalo-bulls, and rams, respectively, compared with the total resolved samples (Table 1). The specificities of the PCR were 99.0%, 100%, and 97.8% while the specificities of culture were 100%, 100%, and 100% in semen samples from bulls, buffalo-bulls, and rams, respectively, compared with the total resolved samples (Table 1). Thirty semen samples were obtained from *Chlamydophila*-free bulls, buffalo-bulls and rams were used as negative control samples and they tested negative by PCR, culture, and nested PCR techniques.

3.2. PCR limit of detection in inoculated semen

To assess the limit of detection of the PCR assay, control bull-semen was mixed with a known number of *C. abortus* (B-577)-IFU subsequently processed for DNA amplification by PCR and culture. A positive PCR result was obtained with different aliquots containing at least 0.25 IFU *C. abortus* per PCR, while direct culture method could detect up to 4 IFU only (Fig. 2). The same result was obtained when *C. pecorum* (11/88) was used by the same manner (gel not shown).

4. Discussion

In this study, a PCR based on the family specific primers and culture techniques were used for the detection of *Chlamydophila* species in semen samples collected from bulls, buffalo-bulls, and rams from farms of known history of infection with *Chlamydophila* spp. We chose a family specific PCR primer pair (Hartley et al., 2001), to amplify the 5′ end of the omp2 gene for all Chlamydiaceae, to enable us to detect all members of the *Chlamydophila* species in semen samples. Because direct PCR detection of most of microorganisms-DNA in semen was difficult and impractical due to the presence of inhibitory components in semen, a protocol was used to extract the DNA from semen samples (Von Beroldingen et al., 1990). Previous experiments using isolation methods suggested that most Chlamydophila agents are present in the seminal fluid and non-sperm cell fractions (Von Beroldingen et al., 1990; Amin, 1993; Amin & More, 1995). The distribution of Chlamydophila-DNA in semen was determined by separating the seminal fluid and non-sperm cell fraction from each naturally infected semen sample (Von Beroldingen et al., 1990) and then analyzed by the PCR assay (Hartley et al., 2001).

PCR assay detected more positive samples (36) from the semen samples collected from different animal species (18 [15%] from bulls, 7 [11.7%] from buffalo-bulls, and 11 [19.6%] from rams) than were detected by the culture method (21) (10 [8.3%] samples of bulls, 4 [6.7%] from buffalo-bulls, and 7 [12.5%] from rams). Out of the 15 PCR-positive, culture-negative samples, 13 were confirmed as true positives and culture failures with an additional nested PCR targeting the omp1 gene (Tong & Sillis, 1993), while two samples were not confirmed and considered false positives. After confirmation, the PCR sensitivity for the detection of *Chlamydophila* species in semen samples collected from different species was 100%, while the culture sensitivities were 58.5%, 57.1%, and 70% in the semen samples from bulls, buffalo-bulls, and rams, respectively, compared with the total resolved samples (Table 1). These differences were significant ($P < 0.005$).

The results indicated that the sensitivity of the PCR was higher than the sensitivity of the culture method. The same conclusion was reached by Domeika et al. (1994) who found that the PCR assay was more sensitive than culture methods for the detection of *C. psittaci* in bull-semen. PCR was also more sensitive than the culture method in detecting *Chlamydophila* species in tissue samples and cultures (Claas et al., 1990; Hewison et al., 1991; Herring, 1993; Amin & More, 1995; Madico et al., 2000; Hartley et al., 2001; Laroucau et al., 2001). This may be attributed to the fact that PCR detects living and dead organisms, since it is based on the detection of genus-specific DNA, while culture methods only detect living organisms. PCR could detect fewer numbers of organisms than could be detected by direct culture. It is note-worthy to mention that no semen samples collected from *Chlamydophila*-free herds were positive by PCR; a finding which indicated satisfactorily the specificity of the assay. The results indicated no significant differences between the specificities of the PCR (99.0%) and culture (100%) techniques (Table 1).

The specificity was tested by adding semen collected from *Chlamydophila* species free-bulls from *Chlamydophila*-free herds to a series of fourfold dilution series of *C. abortus* (B-577) and processing for PCR amplification and culture. The detection limit of *C. abortus* (B-577) in semen was estimated according to the protocols described by Hartley et al. (2001). Positive amplification was always obtained in samples containing at least 0.25 IFU of *C. abortus* (B-577). Culture methods detected when >4 IFU were present (Fig. 2). Similar results were obtained using *C. pecorum* (11/88) (gel not shown). Madico et al. (2000) found the analytical sensitivity of TETR-PCR assay for *C. psittaci* was 0.063 IFU per PCR. The difference in the sensitivity in this study (0.25 IFU per PCR) and in Madico et al.

(2000) study (0.016 IFU per PCR) may be due to the different chlamydial strains and different PCR protocols.

This is the first known report of the application of PCR for detection of *Chlamydophila* species from buffalo-bull semen. The assay has the potential to provide a simple and reliable means for the detection and identification of *Chlamydophila* species from semen samples and has several advantages over the traditional culture method, including speed and enhanced sensitivity. Furthermore, live *Chlamydophila* organisms are not necessary for the assay, which enhances safety and prevents the transmission of the disease to laboratory workers. We recommend the use of the PCR assay as a supplemental diagnostic tool for the detection and identification of *Chlamydophila* species in the semen of different animal species.

References

Aitken, I.D., 1996. Enzootic Abortion of Ewes. OIE Technical series, Office International Des Epizooties, Paris, France.

Amin, A.S., 1993. Studies on Chlamydiosis in farm animals. Ph.D. Thesis, Faculty of Veterinary Medicine, Cairo University.

Amin, A.S., Darwish, G.M., Ziada, M.S., Hassan, H.M., 1999. Trial to control *Chlamydia psittaci* in processed buffalo-semen. Assiut Veterinary Medical Journal 40, 319–331.

Amin, A.S., More, J., 1995. Clinical evaluation of a polymerase chain reaction (PCR) in detection of *Chlamydia psittaci*. In: Proceedings of the Seventh Annual Congress of the Egyptian Society for Animal Reproduction and Fertility, vol. 7, pp. 292–301.

Appleyard, W.T., Aitken, I.D., Anderson, I.E., 1985. Attempted venereal transmission of *Chlamydia psittaci* in sheep. Veterinary Record 116, 535–538.

Beatty, W.L., Morrison, P., Byrne, G.I., 1994. Persistent Chlamydiae: from cell culture to a paradigm for Chlamydial pathogenesis. Microbiological Review 58, 686–699.

Bicknell, E.J., Reggiardo, C., Noon, T.H., Furrey, N., Shupe, M., Mazur, M., Le Viness, E.A., Racicot, R.G., 1986. Diagnostic studies on semen from Arizona range bulls. In: Proceedings of the Annual Meeting of the American Association of Veterinary Laboratory Diagnosticians, vol. 29, pp. 17–24.

Bowen, R.A., Spears, P., Storz, J., Seidel, G.E., 1978. Mechanisms of infertility in genital tract infections due to *Chlamydia psittaci* transmitted through contaminated semen. Journal of Infectious Diseases 138, 95–98.

Claas, H.C.J., Melchers, W.J., Bruijn, I.H., Graaf, M., Dijk, W.C., Lindeman, J., Quint, W.G., 1990. Detection of Chlamydiae in clinical specimens by the polymerase chain reaction. European Journal of Clinical Microbiology and Infectious Diseases 9, 864–868.

Domeika, M., Ganusauskas, A., Bassiri, M., Framan, G., Mardh, A., 1994. Comparison of polymerase chain reaction, direct immunofluorescence, cell culture and enzyme immunoassay for the detection of *Chlamydia psittaci* in bull semen. Veterinary Microbiology 42, 273–280.

Eaglesome, M.D., Garcia, M.M., Stewart, R.B., 1992. Microbial agents associated with bovine genital tract infections and semen. Veterinary Bulletin 62, 899–904.

Everett, K.D.E., Bush, R.M., Andersen, A.A., 1999. Emended description of the order Chlamydiales, proposal pf 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. International Journal of Systemic Bacteriology 49, 415–440.

Fleiss, J.L., 1981. Statistical Methods for Rates and Proportions, second ed. Wiley, New York, NY.

Foote, R.H., 1970. Influence of extender, extension rate, and glycerolating technique on fertility of frozen bull semen. Journal of Dairy Science 53, 1478–1482.

Hartley, J.C., Kaye, S., Stevenson, S., Bennett, J., Ridgway, G., 2001. PCR detection and molecular identification of Chlamydiaceae species. Journal of Clinical Microbiology 39, 3072–3079.

Herring, A.J., 1993. Typing *Chlamydia psittaci*—a review of methods and recent findings. British Veterinary Journal 149, 455–475.

Hewison, R.G., Griffithe, P.C., Rankin, S.E.S., Dawson, M., Woodward, M.J., 1991. Towards a differential polymerase chain reaction test for *Chlamydia psittaci*. Veterinary Record 128, 381–382.

Kaltenboeck, B., Kousoulas, K.G., Storz, J., 1992. Two-step polymerase chain reactions and restriction endonuclease analysis detect and differentiate OmpA DNA of Chlamydia spp. Journal of Clinical Microbiology 30, 1098–1104.

Kwok, S., Higuch, R., 1989. Avoiding false positives with PCR. Nature 339, 237–238.

Laroucau, K., Souriau, A., Radolakis, A., 2001. Improved sensitivity of PCR for Chlamydophila using pmp genes. Veterinary Microbiology 82, 155–164.

Lazono, E.A., 1986. Etiologic significance of bacterial isolates from rams with palpable epididymitis. American Journal of Veterinary Research 47, 1153–1156.

Madico, G., Quinn, T.C., Boman, J., Gaydos, C.A., 2000. Touchdown enzyme time release-PCR for detection and identification of *Chlamydia trachomatis*, *C. pneumoniae*, and *C. psittaci* using the 16S and 16S–23S spacer rRNA genes. Journal of Clinical Microbiology 38, 1085–1093.

Messmer, T.O., Skelton, S.K., Moroner, J.F., Daugharty, H., Fields, B.S., 1997. Application of a nested, multiplex PCR to psittacosis outbreaks. Journal of Clinical Microbiology 35, 2043–2046.

Moulder, J.W., 1991. Interaction of Chlamydiae and host cells in vitro. Microbiological Review 55, 143–190.

Philpott, M., 1994. The dangers of disease transmission by artificial insemination and embryo transfer. British Veterinary Journal 149, 339–369.

Rodolakis, A., Souriau, A., 1986. Response of goats to vaccination with temperature-sensitive mutants of *Chlamydia psittaci* obtained by nitrosoguanidine mutagenesis. American Journal of Veterinary Research 47, 2627–2631.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shewen, P.E., 1986. Chlamydial infection of the bovine reproductive system. In: Morrow, D.A. (Ed.), Current Therapy in Theriogenology. Saunders, Philadelphia, pp. 279–282.

Storz, J., 1971. Chlamydia and Chlamydia-induced diseases. C.C. Thomas, Springfield.

Storz, J., 1988. Overview of animal diseases induced by Chlamydial infections. In: Barron, A.L. (Ed.), Microbiology of Chlamydia. CRC Press, Boca Raton, pp. 167–192.

Storz, J., Carroll, E.J., Ball, L., Faulkner, L.C., 1968. Isolation of a psittacosis agent (Chlamydia) from semen and epididymitis of bulls with seminal vesiculitis syndrome. American Journal of Veterinary Research 29, 549–555.

Storz, J., Carroll, E.J., Stephenson, E.H., Ball, L., Eugster, A.K., 1976. Urogenital infection and seminal excretion after inoculation of

bulls and rams with Chlamydiae. American Journal of Veterinary Research 37, 517–520.

Tong, C.Y.W., Sillis, M., 1993. Detection of *Chlamydia pneumoniae* and *Chlamydia psittaci* in sputum samples by PCR. Journal of Clinical Pathology 46, 313–317.

Von Beroldingen, C.H., Blake, E.T., Higuchi, R., Sensabaugh, G.F., Erlich, H.A., 1990. Applications of PCR to the analysis of biological evidence. In: Erlich, H.A. (Ed.), PCR Technology: Principles and Applications for DNA Amplification. Stockton Press, New York, pp. 209–223.

Walsh, P.S., Metzger, D.A., Higuchi, R., 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10, 506–513.

Watson, P.F., 1990. Artificial insemination and the preservation of semen, fourth ed. Marshall's Physiology of Reproduction, vol. 2. Churchill Livingstone, Edinburgh.