Detection of Ureaplasma diversum in bovine semen straws for artificial insemination

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*Ureaplasma diversum* infection in bulls may result in seminal vesiculitis, balanoposthitis and alterations in spermatozoa. In cows, it can cause placentalitis, fetal alveolitis, abortion and the birth of weak calves (Howard and others 1976, Panangala and others 1981, Miller and others 1983, Pilaszek and Truszczyński 1988, Eaglesome and others 1994, Ruhnke and Rosendal 1994). *U. diversum* is shed in organic secretions, especially semen, preputial and vaginal mucus, conjunctival secretions and milk. Its main transmission route is through direct contact during sexual intercourse or by artificial insemination (Kirkbride 1987, Britton and others 1988, Miller and others 1994). The microorganism infects the uterus via semen or vaginal mucus transmitted from the cervix to the uterus by the insemination pipette (Doig and others 1979), since the vulva can remain infected for at least 60 days after parturition (Miller and others 1983).

Ureaplasmas colonise the prepuce and the distal portion of the urethra in bulls and are rarely isolated from the seminal vesicle and ampulla (Fish and others 1985). The prepuce and urethra may therefore be responsible for semen contamination. Consequently, treatment of the prepuce with topical antibiotics alone for ureaplasma elimination may be ineffective in preventing semen contamination (Doig and others 1980). The addition of antibiotics to semen before it is frozen in straws reduces the number of microorganisms (Fish and others 1985), but no antibiotic treatment regimen has been documented to consistently eliminate ureaplasma contamination in semen. Although several authors have reported the isolation of ureaplasmas from fresh semen (Onoviran and others 1975, Fish and others 1985, Petit and others 2008), data regarding ureaplasma detection in frozen semen are scarce, especially in Brazil. Thus, the aim of the present study was to assess the presence of *U. diversum* in frozen semen straws from breeding bulls in Brazil.

One straw was obtained from each of 35 bulls located at five artificial insemination centres in São Paulo (Brazil). The frozen samples of semen had previously been diluted in a solution containing four antibiotics (per ml of diluent 150 µg lincomycin/300 µg spectomycin and either 500 U/ml penicillin plus 500 U/ml streptomycin, or 50 µg tylosin plus 250 µg gentamicin) according to Normative Instruction 48 from the Brazilian Ministry of Agriculture, Livestock, and Food Supply (Anon 2003).

Straws were stored in liquid nitrogen and transported in dry ice to the laboratory. The samples were cultured in specific liquid and solid media (UB medium) for ureaplasma isolation (Ruhnke and Rosendal 1994). The clinical samples were diluted 10-fold serially (to 10⁻⁰) in 2 ml of liquid media, and 50 µl of each undiluted sample and 50 µl of the 10⁻⁴ dilution were gently streaked on to solid media. Broth and agar cultures were incubated at 37°C aerobically for four weeks and observed daily.

DNA was extracted from the clinical samples (Fan and others 1995). A PCR for detecting Mollicutes was performed using primers described by van Kuppeveld and others (1992), and positive samples were then submitted to a specific PCR to detect *U. diversum* based on a 16S rRNA gene sequence (Cardoso and others 2000).

Ureaplasma colonies were cultured from 13 (37·1 per cent) of the 35 samples and later characterised as *U. diversum* by PCR analyses. The PCR for Mollicutes detected these bacteria in 23 (65·7 per cent) of the 35 samples. *U. diversum* DNA was detected in 17 (48·6 per cent) samples (Fig 1). Samples with a positive culture for ureaplasmas were also positive by PCR analysis for Mollicutes and *U. diversum*.

The frequency of ureaplasma isolation in fresh and treated frozen semen varies worldwide. The frequency observed in the present study was higher than in other studies in which frozen serum was tested. Fish and others (1985) detected *U. diversum* by culture in 19 of 45 (42·2 per cent) fresh semen samples from bulls, but only three samples were positive after the semen had been frozen. Most positive animals (92 per cent) had ureaplasmas in the prepuce in the prepuce, warranting more thorough lower abdomen and preputial cleaning during collection. On the other hand, Onoviran and others (1975) isolated *U. diversum* from six of 42 (14·3 per cent) frozen semen samples, whereas 34 of 140 (24·3 per cent) fresh semen samples presented this microorganism. In other studies, the frequency of *U. diversum* detection in stored semen samples not treated with antibiotics varied from 23 per cent to 84 per cent (Taylor-Robinson and others 1969, Le Grand and others 1995).

*U. diversum* infection in bulls is frequently asymptomatic (Mulira and others 1992), and a high prevalence of *U. diversum* was observed in the present study. The standard antibiotic mix used therefore does not appear to prevent the presence of viable organisms in frozen-thawed semen, and frozen semen presents a risk of transmission of the organism to inseminated female cattle. Clinical data for the studied animals were not available; nevertheless, the animals were considered to be healthy at the time of semen sampling. Additionally, antibiotics currently added to semen diluents are ineffective against Mollicutes (Cardoso and Vasconcellos 2004), indicating that control must rely on sanitary measures.

**FIG 1:** Electrophoresis of PCR products with primers to detect *Ureaplasma diversum* in frozen bovine semen straws. Lanes 1 to 13 Frozen semen straw samples; lane 14 Positive control (*U. diversum*); lane 15 Negative control with water; lane 16 Molecular size markers of 100 bp. Lanes 2, 3, 6 and 12 show positive results

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216 bp

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<td>1</td>
<td>Frozen semen straw samples</td>
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<tr>
<td>2</td>
<td>Positive control (<em>U. diversum</em>)</td>
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<tr>
<td>3</td>
<td>Negative control with water</td>
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<td>6</td>
<td>Molecular size markers of 100 bp</td>
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<td>Molecular size markers of 100 bp</td>
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procedures and hygiene, including animal segregation, as well as novel treatment protocols generated by research based on Brazilian strains.

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