Campylobacter and Tritrichomonas Diagnostic Methods – A summary

Campylobacter fetus venerealis (CFV)

Culture

CFV can be isolated from the genital tract of cattle via preputial smegma and vaginal mucus, or from the internal organs of aborted foetuses (OIE 2008). The organism can be identified when cultured at 37° for at least 3 days micro aerobically in a transport rich media (Givens 2006). The organism is fastidious and requires a complex support medium in addition to antimicrobials to suppress the growth of commensals and contaminants (BonDurant 2005). However it should be noted that bacterial culture of preputial and vaginal washings for Vibriosis have relatively poor sensitivity (Truyers et al. 2014), and should be combined with PCR or Serological testing to minimise the number of false negatives. Differential diagnosis between CFV and *Campylobacter fetus fetus* (CFF) is difficult and unreliable, and biochemical typing, ELISA, direct immunofluorescence or molecular based assays such as PCR are required to differential between the two (McGoldrick et al. 2013).

Biochemical Phenotyping

Following identification of typical Campylobacter colonies, further biochemical phenotyping is require to determine the subspecies present. Both CFV and CFF are catalyse positive, and glycine tolerance testing, as well as H_2S production tests are required to differentiate them. CFV is negative to both tests (BonDurant 2005). Traditional phenotyping is time consuming and has been found to only correctly identify 80% of *C fetus* strains, compared to alternate testing like PCR which correctly identified 98% (Hum, Quinn and J 1997).

Vaginal Mucus Agglutination Test (VMAT)

VMAT was previous employed by diagnostic laboratories to measure the amount of specific IgA activity in the cranial vaginal mucous of cattle. Results were obtained using a vaginal tampon and determine the weight gained following insertion into the vagina for several minutes and reported as the agglutination activity per gram of mucus. This test has reportedly low sensitivity and is more useful for herd screening rather than for diagnostics for individual animals. Unfortunately this test is also limited as the local IgA response can continue for several months following exposure and are unhelpful in determining the current infection status.

Serology - Enzyme Linked Immunosorbent assay (ELISA)

Due to difficulty diagnosing bovine venereal campylobacteriosis, it is suspected to be underrated as a cause of infertility. An enzyme linked immunosorbent assay (ELISA) test has been developed, which detects IgA antibodies present in the vaginal mucus (Hum, Quinn and Kennedy 1994). This has a reported sensitivity of 98.5% in Northern Australian Cattle, and displays the results as positive, inconclusive, suspect (low positive) and negative (McGowan et al. 2014). The results of an immunoassay should include diagnostic possibilities, based on test performance data related to large populations of known disease status and in consideration of actual disease prevalence in representative reference populations Hum, Stephens and Quinn 1991), unfortunately this data is not available as yet for Tasmanian cattle.

The standard sampling is simple and independent of the oestrous cycle, avoiding mucus processing problems associated with VMAT (Hum, Stephens and Quinn 1991).The presence of positive reactors is useful in providing an estimate of the risk of vibriosis affecting the herd reproductive performance.

Unfortunately false positive reactions are possible due to the antibody fluctuation within individual animals (Truyers et al. 2014), and is limited by its inability to distinguish between CFV and CFF subspecies of Campylobacter (OIE 2008).

Polymerase Chain Reaction (PCR)

Each subspecies has its own unique pattern of DNA bands on an agarose gel that are displayed after electrophoresis and can be used for distinguishing CFV and CFF. CFV has a high sequence identity shared with CFF, but there are some unique identifiers (Michi et al. 2016). Differentiating characteristics include a pathogenicity island and mobility genes such as insertion sequence transposase, and these have been used in the design of several PCR primer sequences. The most reliable and quantitative being Primer MG3F/MG4R for the carbon starvation protein gene found in both *C fetus* subspecies and the Primer VenSF/VenSR, a fragment of the parA gene which is exclusive for CFV (BonDurant 2005).

PCR may be combined with other molecular techniques in order to increase its sensitivity, such as restriction fragment length polymorphism (RFLP) or amplification (BonDurant 2005). Due to the recent publication of the complete genome of CFV, improved diagnostics such as real time PCR have been developed allowing for detection of *C fetus venerealis* directly from clinical samples, limiting the need to perform prior culturing (Hum, Hornitzky and Berg 2009). McGoldrick et al. (2013) successfully developed two real-time assays that have a reported sensitivity of 98.7% and specificity for 98.7% for the detection of CFV.

Tritrichomonas foetus (T foetus)

Culture

T foetus is often diagnosed by cultivation of live organisms from reproductive secretions, in selected media such as Diamond's or InPouch (Grahn et al. 2005). The culture is incubated at 37° for several days with regular light microscopy observations. The protozoan is identified based on its morphology and characteristic motility, which has the ability for false positives due to low specificity (Sp) and identification of other Trichomonads such as *Tetratrichomonas* spp (Michi et al. 2016; Mukhufhi et al. 2003). Culturing is time consuming (7-10 days), has low sensitive (Se) in field conditions and is prone to contamination with other trichomonadid (intestinal or coprophilic), and as such a better diagnostic test is essential (Mutto, Giambiaggi and Angel 2006; Grahn et al. 2005).

The current 'gold standard' is six weekly cultures, to confirm the absence of *T foetus* in breeding bulls using smegma (Michi et al. 2016). This has a Se of 86.7% and Sp of 95.4% (Cobo et al. 2007). Repeated sampling is required due to low sensitivity, that is highly dependent on sampling procedures including collection method, transport medium, holding temperature and time delay till processing (Mukhufhi et al. 2003). Time being the most important factor, with a decline in viable organisms being documented by several studies of *T foetus* in culture (Reece, Dennett and Johnson 1983).

Electron Microscopy

Although light microscopy is often used for the preliminary identification of *T foetus*, diagnosis is limited by the possibility of false positives with the identification of other tricomonad organisms. *T foetus* has a characteristic morphology and 'jerky' movement that can be used to distinguish it from other trophozoites (Mueller et al. 2015). The protozoan is approximately 5 x 12µ in size, with 3

anterior and 1 posterior flagellae. It has a characteristic undulating membrane along one side with a notable slow, rolling like movement (BonDurant 2005). Electron microscopy (EM) is required to clearly demonstrate the flagellae for identification, however this process is time consuming and more practical diagnostic techniques are required for large numbers of samples.

Serology - Enzyme Linked Immunosorbent assay (ELISA) and Haemolytic Assay

The immune response to trichomonas's is thought to be confined to the lumen of the reproductive tract of females, however weak systemic humoral responses have been demonstrated with experimental intravaginal infection of non-pregnant heifers. A more sensitive, complement mediated haemolytic assay is also available, and can be used for identify females within the 3rd to 4th week after infection. No response to either assay has been reported in bulls. This assay is not offered commercially, and in combination with their limited period of use are not suitable for large scale surveillance (BonDurant 2005).

Polymerase Chain Reaction (PCR)

Diagnostic testing employing both culture and PCR for *T foetus* yields a higher Se and improved Sp, which may be the most cost-effective and practical approach to assessing bulls prior to the breeding season as it will enable the more accurate detection of the protozoan (Michi et al. 2016). Amplification of DNA material by PCR is a potential highly sensitive and specific test that can allow for minute sections of genomic material to be amplified and then detected using specific primers TFR3 and TFR4 (Mukhufhi et al. 2003). PCR can accurately distinguish *T foetus* from other trichomonads, which may be inadvertently detected and interpreted at tritrichomonas via culture and light microscopy.

The only limitation with PCR, is the dramatic decrease in sensitivity (90% at 6 hours to 31% at 5 days), which can result in the failure of detection of trichomonas's if there is any delay in sampling (Mukhufhi et al. 2003; Cobo et al. 2007). If there is to be a delay in transport, it is essential that samples are maintained at 4°C to limit the proliferation of *T foetus* and other organisms collected from the preputial cavity. This limits the ability of the organisms to secrete hydrolytic enzymes which can cause rapid DNA breakdown (Cobo et al. 2007).

Single PCR of preputial fluid washing has a relatively high Se (98.38%) and Sp (93.75%) according to another study by Mutto, Giambiaggi and Angel (2006). Both PCR and culture used in combination had a high sensitivity and the highest specificity (Se 78.3%, Sp 98.5%), and therefore is the most suitable for larger scale surveillance, due to the ease of single sample collection and reduced time-period for results (Appendix I).

Conclusion

Campylobacter fetus veneralis

Bovine venereal campylobacteriosis is of significant economic importance to the livestock industry, and as such the detection and discrimination of CFV from CFF is essential for recognition and control of this bacteria. Currently ELISA can be used to detect antigen specific antibody in vaginal mucous after abortion, however is not suitable for use in bulls and current sensitivity and specificity relating to Tasmanian cattle in unknown. This makes it unsuitable for regular surveillance, however it is useful for investigating bovine abortion. The development of real time PCR for CFV, allows for quick, repeatable sampling with high sensitivity and specificity. Primers can easily be acquired and allow for PCR to be considered as a possible diagnostic tool for large scale CFV screening of Tasmanian herds.

Tritrichomonas foetus

A testing strategy for *T foetus* diagnostics, consisting of culture and PCR, may be suitable for surveillance. This strategy has a similar sensitivity and improved specificity to current gold standard of six weekly cultures, and is beneficial in regards to reduced cost and time required (Cobo et al. 2007). If time is limited, a single PCR on preputial fluid could be used for surveillance as this also has relatively high sensitivity and specificity, and can be realistically carried out using the facilities of a standard laboratory of clinical analysis (Mutto, Giambiaggi and Angel 2006). The Animal Health Laboratory (AHL) of Launceston has recently acquired primers for *T foetus* testing, although they are yet to test it, its availability allows for its future utilization in surveillance programs. Previous testing required samples to be sent to a laboratory in Queensland, which with the added delay in transport can result in drastically reduced sensitivity of testing.

Reproductive Surveillance

A 2015 pilot study on the prevalence of Vibriosis and Trichomoniasis in Tasmania Cattle Herds had reportedly few samples submitted in for testing, however a Meat and Livestock Australia (MLA) funded survey of Tasmanian Beef herds identified a significant fertility problem possibly caused by CFV and/or *T foetus* (Sherriff et al. 2014). Over the last 17 years a total of 11 confirmed cases of Campylobacterosis and 1 confirmed and 1 suspicious case of Trichomoniasis have been documented from Animal Health Laboratory (AHL) in Launceston.

Currently there is no routine reproductive surveillance on natural breeding Tasmania beef and dairy herds for these two venereal diseases. Results obtained by AHL are limited to those samples sent in by local veterinarians who are not performing regular screening of breeding bulls. Current evidence from confirmed cases submitted in for testing at AHL, suggest that CFV is still present within Tasmanian beef and dairy herds, with some sporadic cases of Trichomoniasis as well. This indicates the need for more regular surveillance, with the inclusion of preventative programs such as annual vaccinations and the culling of older bulls and repeatedly empty or late calving cows.

If sample quality and quantity is adequate, and the sample is rapidly transport and processed by a lab, PCR is the most ideal testing option. The use of real-time PCR for both *T foetus* and CFV, can allow for detection of PCR amplification during the early phases of the reaction, compared to tradition methods of detection at final phase. This allows for the quick identification of the two organisms with high Se and Sp, in comparison to culture, which may prove useful for large scale investigations into poor reproductive performances with Tasmanian cattle herds.

A prevalence survey of Tasmania is recommended, to assess their impact on cattle in the state as a possible cause of low pregnancy testing rates and abortions. If these diseases are identified as present, it is necessary to increase awareness amongst cattle producers and rural veterinarians so that appropriate surveillance can be conduction and that sound preventative measures can be adopted by the industry.

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Appendices

Appendix I: Sensitivity (Se) and Specificity (Sp) and the 95% confidence intervals for *T foetus* culture, PCR, and both methods applied alone, or combined for 2, 3, or 6 weeks. Obtained from a study of bulls experimentally infected with *T fetus* (Cobo et al. 2007).

Test(s)	Week	Se (%)	95% CI	Sp (%)	95% CI
Cult	**1**	**67.8**	51.1; 84.1	**98.8**	96.3; 101.2
PCR	**1**	**65.9**	49.5; 81.8	**98.3**	95.0; 101.6
Both	**1**	**78.3**	63.7; 93.0	**98.5**	95.6; 101.4
Cult	**2**	**76.0**	60.8; 91.3	**98.5**	95.6; 101.4
PCR	**2**	**78.0**	64.0; 92.0	**96.7**	91.6; 101.9
Both	**2**	**83.3**	70.2; 96.5	**96.5**	90.9; 102.1
Cult	**3**	**80.0**	65.8; 94.2	**98.1**	94.5; 101.8
PCR	**3**	**85.0**	72.3; 97.7	**95.4**	89.6; 101.2
Both	**3**	**87.5**	75.9; 99.1	**95.6**	9.4; 101.9
Cult	**6**	**86.7**	74.5; 98.8	**97.5**	92.7; 102.3
PCR	**6**	**90.0**	79.3; 100.7	**91.8**	84.2; 99.5
Both	**6**	**93.3**	84.4; 102.3	**92.5**	84.3; 100.7

Bulls considered positive had to have a culture and/or PCR positive results on their smegma samples at least once during the 6-week period. Bulls considered negative had to have culture and PCR negative results on all of their samples.