Ministry for Primary Industries
Manatū Ahu Matua

Discussion Document

Mycoplasma bovis Risk Management in Bovine Germplasm

BOVEMID.GEN and BOVSEMID.GEN

24 September 2019

Growing and Protecting New Zealand

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1 Purpose

The import health standards for bovine germplasm are currently being updated in line with current MPI policies and processes, whereby the measures for all the diseases managed are being reviewed and updated in line with latest science and risk analysis.

Following the detection of *Mycoplasma bovis* (*M. bovis*) in 2017, MPI and New Zealand industry initiated a national eradication program with a commitment of some $886 million of industry and crown expenditure. This has led to a prioritisation of the review of risk management options for *M. bovis* and a decision to carry out a special, non-statutory, public consultation on the science, the risk assessments, the risk management options, and the feasibility of their application to effectively manage the biosecurity risks associated with *M. bovis* contamination of bovine semen and embryos.

This document outlines the risk management options available for *M. bovis* in frozen semen and embryos. It seeks stakeholder comment on the scientific justification of the measures, and the feasibility of application as regulatory requirements by:

- Consulting on the *Mycoplasma bovis* in bovine semen – Rapid Risk Assessment (RRA) and the *Mycoplasma bovis* in bovine in-vivo derived and in-vitro produced embryos – Rapid Risk Assessment.
- Reviewing the current import requirements (issued in 2010).
- Describing the available methods to reduce the risk of *M. bovis* in bovine semen and embryo imports and the uncertainties associated with each method.
- Presenting potential new risk management options for *M. bovis* in bovine semen and embryo imports.

This consultation is in addition to a future consultation of the Import Health Standard (IHS), if it is proposed for amendment.

2 International Obligations

This discussion document is to be read with full consideration of New Zealand's obligations under our international agreements.

The World Trade Organisation *Agreement on the Application of Sanitary and Phytosanitary Measures* (the SPS Agreement) specifies that all measures must be based on risk assessment and sound science, consider relevant epidemiological and economic factors, minimise negative trade effects and that the government not introduce discriminatory measures where similar conditions prevail, including between that country and that of other Members.

Any future decision to amend the IHS will require the CTO to consider all obligations of the Biosecurity Act, which includes consistency with the SPS and WTO obligations.

3 Background

Bovine semen and embryos have the potential to harbour exotic viral and bacterial diseases. In 2009 the Ministry for Primary Industries (MPI) (at the time known as the Ministry of Agriculture and Forestry, MAF) completed the 2009 import risk analysis (IRA) for bovine germplasm. In 2010 work commenced on developing a generic import health standard (IHS) based on that analysis.

The draft IHS developed in 2010 contained generic import requirements, including a testing requirement for *M. bovis.* The organism is not OIE listed and is therefore not covered by a chapter in the OIE *Code.* The chapters that cover semen and embryo hygiene, collection, processing, and storage do not provide risk management recommendations specific to *M. bovis,* but they contain measures that provide some effect against *Mycoplasma* spp in general. Further, some countries impose specific protocols to manage *M. bovis* in semen. Those protocols are discussed in Section 7 of this document.

In regard to guidelines for embryos, the International Embryo Technology Society (IETS) provides a significant amount of information about research that has been done to assess the feasibility of removing *M. bovis* from embryos. The recommendation IETS provides is based upon a study conducted in 1993.

The *M. bovis* requirement in the 2010 IHS included donor serological testing. During public consultation, New Zealand's trading partners noted that it was not an OIE listed disease, and questioned New Zealand's country freedom claim, the transmissibility of *M. bovis* in germplasm and the economic impact of the disease should it enter New Zealand. It was requested MPI remove the test requirement.

At that time, MPI presented trading partners with the results of a 2007 survey which used bulk tank milk testing by PCR and culture. The sampling was designed to detect a herd prevalence of above 2% with 99% confidence. There were no positives, suggesting the New Zealand dairy industry, at least, was either free of *M. bovis* or if it was present it was at a very low prevalence.

MPI acknowledged that published data demonstrating the transmission of *M. bovis* via insemination of commercially collected germplasm was lacking and this conclusion was supported by the history of imports into New Zealand of germplasm over many years with no apparent *M. bovis* introduction.

New Zealand's trading partners provided submissions that claimed that their antibiotic protocols are effective against mycoplasmas.

The Bovine Semen IHS and Bovine Embryo 2010 IHSs were revoked, amended and reissued in 2011, with a simplified *M. bovis* requirement that the donor has never recorded a positive test. The IHSs include the requirements of the OIE *Code* Chapters 4.6, 4.7, and 4.8, and, to align with the European Union (EU), semen has to be held at a temperature of at least 5 °C for a period of not less than 45 minutes before freezing.

Country to country (bilateral) negotiations of country specific veterinary certificates began in 2011 using the 2011 IHS.

4 The investigation into the *M. bovis* incursion

In July 2017 *M. bovis* was discovered in cattle on a South Island dairy farm, prompting the initiation of an incursion response. Subsequently, the organism was traced throughout New Zealand; however, as of May 2019 all new cases continue to be linked by molecular analysis to a single incursion. Gene sequencing supports this conclusion and DNA mutation analysis suggests the time of the incursion was 2015.

A pathways report was produced in 2017 and bovine semen and embryos were speculated to be the most likely pathways in 2015 due to the absence of live cattle imports since 2013. However, a complex analysis of semen used on infected properties was conducted and no clear link between the current outbreak and semen imports has been found.

In December 2017, the rapid risk assessment: *M. bovis* in semen (RRA) was published. It was updated and reissued in 2019. The risk of entry was assessed as low. Then in 2018 the rapid risk assessment: *M. bovis* in in-vivo and in-vitro derived embryos was published. The risk of entry was assessed as very low.

In both RRAs, the likelihood of transmission is assessed to be very low, but non-negligible. The overall risk estimate is non-negligible. It is stated that risk management may be warranted. This was the same conclusion that was reached in the 2009 Risk Analysis.

The RRAs included a review of a transmission study, published by Haapala et al. in early 2018[1]. The review concluded that identifying processed semen as the source of the outbreak was problematic due to *M. bovis* being endemic in Finland and the lag time between the use of infected semen and clinical diagnosis. Nevertheless, this

[1] Haapala V, Pohjanvirta T, Vahanikkila N, Halkilahti J, Simonen H, Peokonen S, Soveri T, Simojoki H, Autio T. "Semen as aa source of *Mycoplasma bovis* mastitis in dairy herds", Veterinary Microbiology, 2018, (216) 60-66

is the most conclusive study to date to demonstrate the infected semen processed according to international standards might cause an outbreak of disease. As a result, MPI has more evidence now than in 2011 that semen with viable *M. bovis* may pose a risk of transmission, however that risk is still considered very low.

As mentioned in the bovine semen RRA, if semen transmission were a frequent international event (i.e. not very low risk), then a greater diversity of strains would be seen around the world, or at least in countries that import semen.

A final relevant point is that the strain of *M. bovis* in New Zealand has preliminarily shown susceptibility to routine antimicrobials used in semen preparations. It is therefore less likely that antibiotic resistance, and therefore legally imported semen, was the cause of New Zealand's first incursion.

5 Eradication

On 28 May 2018, the New Zealand Government, along with the dairy and beef industries, made the decision to attempt to eradicate *M. bovis*. Together they have committed $886 million for a 10 year eradication programme. At that time extensive surveillance and testing had demonstrated a limited distribution and a linked network of infected farms and genotyping indicated there was a single point incursion around mid 2015. As of 18 September 2019, there have been 193 Confirmed Properties (CP), 114,736 animals culled and 913,985 tests completed. Updates on surveillance and testing can be found in the *M. Bovis* situation report.

A Spring Surveillance Programme using bulk milk testing (BMT) for the country's 12,000 dairy herds commenced in July 2018 and concluded in April 2019. This detailed survey of dairy properties nationwide was designed to screen milking herds during the stressful time post-calving when an increased circulation of *M. bovis* is expected. Spring milk samples were taken starting four weeks after the start of lactation, and then collected every two weeks for a 12 week period (six PCR samples; one every fortnight, and three ELISA samples; one every month). Potential infected properties (IPs) identified from the BMT programme are then subjected to individual animal testing and if confirmed positive the herd will be culled.

The results of the Spring Surveillance Programme are an indication of the extent of infection in the dairy sector. ELISA and PCR tests for *M. bovis* do not have perfect performance. The results of the Spring Surveillance Programme indicate an apparent prevalence in dairy herds of 0.55% (99% CI: 0.39%-0.75%) over the period[2]. The small number of newly detected herds following this Programme supports the assumption that there is not widespread, unlinked disease in the dairy herd population. Given the data, there has been growing confidence that eradication is achievable.

A survey involving 205 calf rearing properties that had no identified connection with any infected properties was initiated in October 2018. Bull dairy calves in New Zealand are reared on beef farms and make up 19% of the adult cattle at slaughter[3]. The intention of the survey was to support or challenge the theory of a single point incursion. Testing involved nasal swabbing of calves on randomly selected calf rearing properties across New Zealand. No positives were found. To date the only infections on beef properties have been connected by animal movements, supporting evidence of a single point incursion.

In immediate response to farmers' concerns over semen possibly being the source of the outbreak and their desire to see specific *M. bovis* management within the domestic bovine semen supply, the major supplier started adding enrofloxacin (at 5ppm) to the semen diluent for liquid semen for domestic use only, testing donors by PCR (tonsil and preputial swab), and testing semen batches by PCR. Tens of thousands of batches of domestic semen have been tested so far, all yielding negative results.

The eradication programme provides a legal notice (Notice of Direction, NOD) to prohibit all unauthorised movements of farm stock and other risk goods off properties suspected of having *M. bovis*. Once a property is

[2] Communication from the *Mycoplasma bovis* Programme, MPI, 17 May 2019.

[3] https://beeflambnz.com/knowledge-hub/PDF/guide-new-zealand-cattle-farming

issued a NOD, movement and use of semen and embryos from the property requires a permit. At this time, permits cannot be granted for embryos since there are no validated tests and no NODs have been issued on properties where semen is produced.

The eradication programme is ongoing and in June 2019 it was announced that bulk milk tank screening would continue indefinitely. The on-going large scale eradication programme reflects New Zealand's concern over the consequence of *M. bovis* infection in our herds. It indicates that tighter risk management may be appropriate.

6 Semen and embryo collection and processing

The IHS issued in 2011 has the requirement that the donor has never recorded a positive test. This does not indicate that the donor must have been tested. Although that is the only requirement indicated in the IHS for *M. bovis* specifically, MPI's primary risk management has been via the IHS's general hygiene requirements, which includes the primary method of risk management currently in place: antibiotic treatment. The hygiene requirements are described in OIE *Code* Chapter 4.6 and Chapter 4.7 for semen, and 4.8 for embryos.

6.1 The semen hygiene recommendations in Chapter 4.6

- Equipment for use with the livestock should be dedicated to the semen collection facilities or disinfected prior to entry. All equipment and tools brought on to the premises should be examined and treated if necessary to ensure that they cannot introduce disease.
- The semen laboratory should be physically separated from the semen collection facilities, and include separate areas for artificial vagina cleaning and preparation, semen evaluation and processing, semen pre-storage and storage. Entry to the laboratory should be prohibited to unauthorised personnel.
- Only semen collected from donors having a health status equivalent to or better than the donors at the semen collection facilities should be processed in the laboratory.
- The coat of the animal should be kept clean.
- For bulls, the tuft of hairs at the preputial orifice, which is often soiled, should be cut to about 2 cm. The hair should not be removed altogether, because of its protective role. If cut too short, irritation of the preputial mucosa may result because these hairs aid the drainage of urine.

6.2 The semen recommendations in Chapter 4.7

- The hindquarters of the teaser, whether a dummy or a live teaser animal, should be kept clean. A dummy should be cleaned completely after each period of collection. A teaser animal should have its hindquarters cleaned carefully before each collecting session. The dummy or hindquarters of the teaser animals should be sanitized after the collection of each ejaculate. Disposable plastic covers may be used.
- The hand of the person collecting the semen should not come into contact with the animal's penis. Disposable gloves should be worn by the collector and changed for each collection.
- The artificial vagina should be cleaned completely after each collection where relevant. It should be dismantled, its various parts washed, rinsed and dried, and kept protected from dust. The inside of the body of the device and the cone should be disinfected before re-assembly using approved OIE approved techniques such as those involving the use of alcohol, ethylene oxide or steam. Once re-assembled, it should be kept in a cupboard which is regularly cleaned and disinfected.
- The lubricant used should be clean. The rod used to spread the lubricant should be clean and should not be exposed to dust between successive collections.
- The artificial vagina should not be shaken after ejaculation, otherwise lubricant and debris may pass down the cone to join the contents of the collecting tube.
- When successive ejaculates are being collected, a new artificial vagina should be used for each mounting. The vagina should also be changed when the animal has inserted its penis without ejaculating.
- The collecting tubes should be sterile, and either disposable or sterilised by autoclaving or heating in an oven at 180°C for at least 30 minutes. They should be kept sealed to prevent exposure to the environment while awaiting use.

- After semen collection, the tube should be left attached to the cone and within its sleeve until it has been removed from the collection room for transfer to the laboratory.

6.2.1 Diluents

- All receptacles used should have been sterilised.
- Buffer solutions employed in diluents prepared on the premises should be sterilised by filtration (0.22 μm) or by autoclaving (121°C for 30 minutes) or be prepared using sterile water before adding egg yolk (if applicable) or equivalent additive and antibiotics.
- If the constituents of a diluent are supplied in commercially available powder form, the water used should have been distilled or demineralised, sterilised (121°C for 30 minutes or equivalent), stored correctly and allowed to cool before use.
- Whenever milk, egg yolk or any other animal protein is used in preparing the semen diluent, the product should be free from pathogenic agents or sterilised; milk heat-treated at 92°C for 3–5 minutes, eggs from SPF flocks when available. When egg yolk is used, it should be separated from eggs using aseptic techniques. Alternatively, commercial egg yolk prepared for human consumption or egg yolk treated by, for example, pasteurisation or irradiation to reduce bacterial contamination, may be used. Other additives should also be sterilised before use.
- Diluent should not be stored for more than 72 hours at +5°C before use. A longer storage period is permissible for storage at -20°C. Storage vessels should be stoppered.
- A mixture of antibiotics should be included with a bactericidal activity at least equivalent to that of the following mixtures in each ml of frozen semen: gentamicin (250 μg), tylosin (50 μg), lincomycin–spectinomycin (150/300 μg); penicillin (500 IU), streptomycin (500 μg), lincomycin-spectinomycin (150/300 μg); or amikacin (75 μg), divekacin (25 μg). The names of the antibiotics added and their concentration should be stated in the international veterinary certificate.

6.2.2 Procedure for dilution and packing

- The tube containing freshly collected semen should be sealed as soon as possible after collection, and kept sealed until processed.
- After dilution and during refrigeration, the semen should also be kept in a stoppered container.
- During the course of filling receptacles for dispatch (such as insemination straws), the receptacles and other disposable items should be used immediately after being unpacked. Materials for repeated use should be disinfected with alcohol, ethylene oxide, steam or other approved disinfection techniques.
- If sealing powder is used, care should be taken to avoid its being contaminated.

6.3 The embryo recommendations in Chapter 4.8

- The embryo processing laboratory should be under the direct supervision of the team veterinarian and be regularly inspected by an Official Veterinarian.
- While embryos for export are being handled prior to their storage in ampoules, vials or straws, no embryos of a lesser health status should be processed.
- Only embryos from the same donor should be washed together, and no more than ten embryos should be washed at any one time.
- Any biological product of animal origin used in the media and solutions for collection, processing, washing or storage of embryos should be free from pathogenic agents. Media and solutions used in the collection and storage of embryos should be sterilised by approved methods in accordance with the IETS Manual and handled in such a manner as to ensure that sterility is maintained. Antibiotics should be added to collection, processing, washing and storage media as recommended in the IETS Manual.
- All equipment used to collect, handle, wash, freeze and store embryos should ideally be new or at least sterilised prior to use as recommended in the IETS Manual.
- Used equipment should not be transferred between countries for re-use by the embryo collection team.

6.3.1 Donor animals

- The Veterinary Authority should have knowledge of, and authority over, the herd from which the donor animals have been sourced.
- The donor animals should not be situated in a herd subject to veterinary restrictions for OIE listed disease or pathogenic agents for relevant species (see Chapter 1.2.), other than those that are in International Embryo Technology Society (IETS) Category 1 for the species of embryos being collected (see Article 4.8.14.).
- At the time of collection, the donor animals should be clinically inspected by the team veterinarian, or by a veterinarian responsible to the team veterinarian and certified to be free of clinical signs of diseases.

6.3.2 Semen Donors

- Semen used to inseminate donor animals artificially should have been produced and processed in accordance with Chapter 4.7.
- When the donor of the semen used to inseminate donor females for embryo production is dead, and when the health status of the semen donor concerning a particular infectious disease or diseases of concern was not known at the time of semen collection, additional tests may be required of the inseminated donor female after embryo collection to verify that these infectious diseases were not transmitted. An alternative may be to test an aliquot of semen from the same collection date.
- Where natural service or fresh semen is used, donor sires should meet the health conditions set out in Chapter 4.7 as appropriate to the species.

Hygiene requirements could be considered important to risk management because *Mycoplasma* spp., including *Mycoplasma bovis* specifically[4], are known to be a contaminant from the prepuce and urethra[5], which are in contact with the collection equipment. It is unknown how often *M. bovis* can be considered purely a surface contaminant (i.e. not also present in the ejaculate). *M. bovis* has been cultured from samples taken directly from the upper genital tract, albeit infrequently[5]. For this reason, proper hygiene alone would not manage the risk, but it could be considered important in helping to reduce the overall bacterial count.

The OIE *Code's* recommendations do not include cleaning any portion of the genital tract. Effective cleaning of the urethral orifice and prepuce could help bring the bacterial count below the infective dose (which is currently unknown) and within the range that the antibiotic combination is expected to manage (Shin[6] concluded effective management with inoculation at10^5 -10^6 cfu/ml and effective management at 10^3 cfu/ml was demonstrated by Pohjanvirta[7]). There may be value in cleaning the prepuce and urethral orifice specifically; however, the feasibility and the efficacy of cleaning are yet to be demonstrated.

[4] Kissi B, Juhasz S, Stipkovits L, "Effect of Mycoplasma contamination of bull semen on fertilization" Acta Veterinaria Hungaria 1984. 33: (1- 2).

[5] Fish NA, Rosendal S, Miller RB, "The distribution of mycoplasmas and ureaplasmas in the genital tract of normal artificial insemination bulls", The Canadian veterinary journal, 1985. 26(2), 321-3.

[6] Shin SJ, Lein DH, Patten VH, Ruhnke HL, "A New Antibiotic Combination for Frozen Bovine Semen. 1. Control of Mycoplasmas, Ureaplasmas, Campylobacter fetus subsp. venerealis and Haemophilus somnus". Theriogenology 1988. 29:577.

[7] Pohjanvirta T, Vahanaikkila N, Simonen H, Pelkonen S, and Autio T, "The effect of different extender protocols on viability of *Mycoplasma bovis* in bovine semen", European Mycoplasma Conference, 2019. Poster. See appendix 1.

6.4 Processing

A process for the physical removal of bacteria from semen is called Single Layer Centrifugation (SLC) and success has been shown for some bacterial species (Morrel and Wallgren[8]), however, *M. bovis* adheres to sperm cells and so this type of process would not be expected to remove *M. bovis* from semen.

It has been hypothesised that semen infected with *M. bovis* may be of poor quality and during processing sperm should be evaluated. An increased number of dead or morphologically abnormal spermatozoa and reduced motility of spermatozoa in semen contaminated with *Mycoplasmas* has been reported. It is understood that semen is routinely evaluated prior to export as a matter of quality control. A 2018 study by Hazelton[9] showed that subclinical semen donors did not have poor semen quality attributes such as semen mass activity, sperm motility, and morphology. Therefore, there does not appear to be any rationale for requiring semen evaluation. It is unknown if *M. bovis* affects the quality of embryos, and *M. bovis* cannot be visualised on the surface of an embryo. Therefore, additional evaluation of embryos is not recommended.

MPI examined the current antibiotics recommended by the OIE *Code* and required as per the bovine semen and embryo IHSs. As seen above in the excerpt from Article 4.7.7, the OIE *Code* provides three antibiotic combination options (antibiotic dose is per mL extended semen):

1) Gentamicin (250 μg), tylosin (50 μg), lincomycin–spectinomycin (150/300 μg) (GTLS);
2) Penicillin (500 IU), streptomycin (500 μg), lincomycin-spectinomycin (150/300 μg);
3) Amikacin (75 μg), divekacin (25 μg).

Because of the lack of cell wall and its metabolic and nutritional differences from other bacterial agents, *M. bovis* is naturally resistant *to* β-lactam antimicrobials (such as penicillin) and sulphonamides. There are many studies showing *Mycoplasma* spp have become quite resistant to the aminoglycosides (including gentamycin, streptomycin, divekacin/dibekacin and amikacin). Resistance to fluoroquinolones is increasing. It appears *M. bovis* is most sensitive (or less resistant) to macrolides (tylosin), spectinomycin (an aminocyclitol, which is similar to the aminoglycosides), tetracyclines and lincosamides. The tetracyclines are considered to have toxic effects at low concentrations and resistance to fluoroquinolones has been increasing (as discussed in the bovine semen RRA). The bovine semen RRA indicated increasing minimal inhibitory concentrations (MICs) of macrolides. Of significance was the observation in 2014 that tylosin had a MIC90> 128ug/ml and yet the OIE only recommends tylosin at 50 μg/ml in the GTLS combination.

It appears that among the options in the OIE *Code* the GTLS option could be considered the best. As there doesn't appear to be a study comparing the combinations, looking at their individual profiles is the most scientific approach at this time.

While Certified Semen Services (CSS) of the USA has a specific protocol in place for GTLS[10], that does not appear to be true for our other trading partners. The OIE *Code* also does not require anything specific about the process to treat semen or embryos with antibiotics, not even to specify the length of time recommended from addition of antibiotics until freezing. Semen processing protocols may have varying impacts on the efficacy of the antibiotics. For example, changing the glycerol equilibration time or the order in which the extender/cryoprotectant/antibiotics are added would be expected to change the degree to which antibiotics cross the *M. bovis* cell membrane.

[8] Morrel JM, Wallgren M, "Alternatives to Antibiotics in Semen Extenders: A Review", Pathogens 2014 3(4): 934–946.

[9] Hazelton MS, Morton JM, Bosward KL, Sheehy PA, Parker AM, Dwyer CJ, Niven PG, House JK. "Isolation of Mycoplasma spp. and serological responses in bulls prior to and following their introduction into Mycoplasma bovis-infected dairy herds". J Dairy Sci. 2018 Aug;101(8):7412-7424

[10] https://www.naab-css.org/uploads/userfiles/files/CSSMinReq-Jan2014201607-ENG.pdf

It has been demonstrated by Ahmad[11] that different combinations of antibiotics and extenders alter semen fertility. The results of that work appear to have influenced the requirements around extenders in the CSS Minimum Requirements (e.g. permitting a 1-step method for 20% Egg Yolk Tris extender). The OIE hygiene requirements focus only on extenders as a potential source of contamination. It remains unclear if extenders affect antibiotic efficacy or bacterial cells as much as they affect fertility (i.e. sperm cells).

It is noted that CSS provides two methods for antibiotic treatment (1-step and 2-step). The 2-step method produces a final concentration of 50 μg tylosin, 250 μg gentamicin and 150/300 μg of Linco-Spectin in each ml of frozen semen. The final concentration of antibiotics in the 1-step method is essentially doubled compared to the standard 2-step method. The difference between the two methods is that the 1-step method uses an extender that is not fractionated into a non-glycerol and glycerol component. The concentration of GTLS recommended by the OIE is the same as that prescribed for GTLS 2-step method.

Both methods involve a 3-5 minute neat semen antibiotic treatment before any extender is added. In both methods, the ultimate glycerol concentration is 7% and cooling with antibiotics to 5 °C must be for a period of not less than two hours. CSS methods vary only slightly from the methodology described by Shin et al.; CSS methods do not require glycerol equilibration by holding the fully extended semen at 5 °C for four hours before freezing. It is unknown how glycerol equilibration may affect the antibiotic treatment, but semen producers likely choose to hold it for an extended period to attain the full cryoprotective benefit from glycerol. There appears to be no justification for adding a glycerol equilibration time requirement.

In 1988 Shin el al. did not declare that their protocol afforded a 100% bactericidal effect, but rather effective *Mycoplasma* control through majority bacteriostatic effect. CSS specifically states that "processing procedures, extender composition, and antibiotic combinations may affect efficacy of microbial control" and they require that their procedures are followed, unless they have CSS approval for a different procedure.

In a study by Lorton and Shin[12] on *Mycoplasma bovis* control in fresh semen, the GTLS treatment was not considered effective. There was no discussion in that study as to why GTLS did not work. The GTLS was used on neat semen, similarly to the frozen semen protocol. There was also no follow up study to confirm that GTLS was still effective under the protocol developed in 1988. Two obvious differences with the fresh semen study was the lack of cryoprotectant, the low temperature during antibiotic treatment, and ultimate freezing. While freezing may help manage the *M. bovis* risk, there is limited evidence. Glycerol is known to be bacteriostatic, but the relatively small amount used in extenders is unlikely to have a significant bacteriostatic effect on *M. bovis*. Glycerol is an intracellular cryoprotector, preventing the formation of ice crystals inside the cells, and is often used in freezing of semen. It is apparent from the two CSS methods that glycerol influences the efficacy of GTLS. The absence of glycerol could be the reason for the lack of *M. bovis* control with GTLS in the Lorton and Shin study. It remains unknown why freezing (with glycerol) is critical in the current CSS protocol. The IHSs specify that the germplasm must be frozen.

The efficacy of GTLS is unclear, and the original studies are decades old. However, in March 2019 Pohjanvirta[13] presented at the European Mycoplasma conference showing that GTLS can (depending upon the technique used) prevent growth of a culture when semen is inoculated with *M. bovis* at 10^3 cfu/ml. However it also showed that GTLS was not effective when the *M. bovis* was inoculated at 10^6 cfu/ml. Interestingly, Pohjanvirta's research demonstrated that antibiotic combinations perform better when they are stored separately and added to extender just prior to use, rather than in the form of ready-to-use extender.

According to Pohjanvirta, there is no advantage in adding antibiotics to neat semen, and in one culture it was demonstrated to be less effective than adding antibiotics to the extender before use. While none of the treatments

[11] Ahmad K, Foote RH. "Antibiotics for Bull Semen Frozen in Milk and Egg Yolk Extenders", J Dairy Sci 1987. 70:2439-2443.

[12] Lorton SP, Shin SJ, "Efficacy of antibiotics for liquid bovine semen", published for the New Zealand Embryo Transfer Workshop, 14-15 January 1994, pp. 70-71.

[13] Pohjanvirta et al., "The effect of different extender protocols on viability of *Mycoplasma bovis* in bovine semen", European Mycoplasma Conference, 2019, poster. See appendix 1.

were effective when semen was inoculated at 10^6 cfu/ml *M. bovis*, natural contamination is thought to be at approximately 10^4 cfu/ml. The best antibiotic treatment technique demonstrated by that research is the addition of antibiotics just prior to their use directly into the extender, and this would be the most appropriate processing requirement to stipulate in an amended IHS.

In addition to GTLS, MPI allows the other two antibiotic combinations provided by the OIE *Code*. The OIE Code antibiotic specified in the EU's Council Directive 88/407/EEC for *Mycoplasmas* is*:* Penicillin (500 IU), streptomycin (500 μg), lincomycin-spectinomycin (150/300 μg). The Directive also states that an alternate combination with equivalent effect may be used and that diluted semen is held at a temperature of at least 5°C for a period of not less than 45 minutes. Unfortunately no studies have been found to confirm the effectiveness of the EU antibiotic requirement or the 45 minute holding period. It is understood that the EU has approved GTLS as an antibiotic combination with equivalent effect.

The New Zealand health certificates to import from Canada and Australia specify that one of the three antibiotic options provided by the OIE *Code* is used and that diluted semen is held at a temperature of at least 5°C for a period of not less than 45 minutes.

In regard to embryos, *M. bovis* is considered to adhere tightly to the zona pellucida. Washing has been demonstrated to be ineffective in managing the risk. According to IETS, *Mycoplasma* spp. are also resistant to the addition of tryspin during washing.

The OIE *Code* indicates that antibiotics should be added to collection, processing, washing and storage media as recommended in the IETS Manual. While the OIE *Code* does not stipulate the antibiotics that are required, the IHS indicates that, "Antibiotics as recommended in the OIE *Code* and IETS Manual, or a combination of antibiotics with equivalent activity, must be added to collection, processing, washing and storage media. The names of antibiotics added and their concentration must be stated on the zoosanitary certificate."

The IETS Manual indicates that a 1993 study by Riddell[14] concluded that four hours of incubation at 37°C in media containing tylosin (200 μg/mL) or kanamycin (1000 μg/mL) successfully inactivated *M. bovis* that was associated with in vivo bovine embyros.

In summary, it has become apparent from the literature that *M. bovis* control in semen is possibly effective with GTLS, but its effect is mostly bacteriostatic. Hygiene and antibiotics may keep the number of organisms low, but, upon insemination, the organisms may recover, particularly if the strain is resistant to the antibiotics used. Without ongoing evidence that antibiotics are effectively controlling *M. bovis*, antibiotics alone may not be appropriate risk management.

At this time, because the OIE *Code* recommends the general application of antibiotics for germplasm, and there is some evidence that GTLS is effective for semen and tylosin alone for embryos (as per IETS). MPI could require GTLS at higher than the OIE *Code* recommended concentration (i.e., 100 μg tylosin, 500 μg gentamicin, 300/600 μg Linco-Spectin) justified by the evidence that resistance is increasing and awareness that the 1-step CSS process requires this concentration. MPI could require that germplasm is held for say two hours at a minimum +5°C to potentially get greater effect from the antibiotics. Based upon the results of Pohjanvirta et al., MPI could require specific methods for preparation, storage, and addition of antibiotics to semen. Such requirements might help enhance the efficacy of antibiotic treatment.

[14] Riddell KP, Stringfellow DA, Gray BW, Riddell MG, Galik PK, "Antibiotic treatment of bovine embryos", J Assist. Reprod. Genet, 1993. 10:488-491.

7 Testing for *Mycoplasma bovis*

7.1 Testing germplasm donors and milk for *M. bovis* antibodies

The enzyme-linked immunosorbent assay (ELISA) is a commonly used laboratory based assay designed to detect a protein, by a highly specific antibody-antigen interaction. ELISA therefore detects exposure and infection, rather than shedding. Experimentally, *M. bovis* antibodies can be detected in serum by indirect ELISA 6 to 10 days after infection. However under natural conditions, individual animal titres are not well correlated with infection or disease. As a result of false negatives to ELISA being not uncommon in infected animals, repeat blood ELISA screening is usually necessary.

The ELISA is the only commercially available antibody test for *M. bovis*. The ELISA test is validated as a herd detection tool rather than for use in individual animals. It may be useful in beef herds, or when dairy cattle are not lactating. The ELISA on milk is considered to be a better detection tool for lactating dairy herds.

The bulk tank milk (BTM) ELISA to detect infected herds is considered quite useful. Because the protein demonstrating exposure is not always shed in milk, it is not a perfect herd-level diagnostic tool, although it is considered more likely to return a positive result than milk culture or PCR. The dilution factor of milk reduces the sensitivity of the test, however when a sample from the hospital milk (milk withheld from the bulk tank) is included for testing, the sensitivity improves. The BTM results are considered to be best at detecting infected herds when the test is conducted 5-6 weeks after calving. Repeat BTM ELISAs are recommended and can significantly increase the sensitivity.

The Technical Advisory Group (TAG) provides expert opinion of MPI's response to *M. bovis*. Their January 2019 report provides support for moving the surveillance strategy toward ongoing nationwide monthly BMT by ELISA alone. Repeat BMT ELISAs on embryo donor herds of origin and on collection herds could be considered appropriate risk management for embryos since they are considered very low risk.

7.2 PCR testing

Real-time quantitative PCR (qPCR) is used to detect particular *M. bovis* DNA molecules in a sample. The major challenge to current molecular detection tests is the intermittent shedding of *M. bovis*.

Mycoplasmas are considered to be frequent and insidious inhabitants of mucous membranes of the lower urogenital tract, the tonsils, nasal passages, joints, and milk. PCR testing swabs or samples taken from these locations proves highly sensitive when DNA is present in the tested area.

In regard to milk PCR, sample processing (enrichment) is important. While not as sensitive as ELISA testing milk, PCR of the bulk milk tank is a common screening tool and it is generally considered better than culture. It is best used in conjunction with ELISA milk testing.

Preputial swabs could be easily performed when semen donors are tested for *Campylobacter fetus* subsp. *venerealis* and *Tritrichomonas foetus* as per OIE recommendations (prior to entering the collection centre and annually while resident in the collection centre). Since the prepuce is a known site for semen contamination, preputial swabs might be the best swab location for PCR. Negative PCR on a preputial swab would not be considered as reliable as a negative PCR on a sample of the semen exported to New Zealand.

A validated PCR test is available for semen and it is considered more representative of an ejaculate than a preputial swab PCR test. The test has been used on thousands of semen samples in New Zealand. The Haapala et al. study in 2018 demonstrated inconsistent PCR results, with straws from single ejaculates providing both positive and negative results. Therefore, semen should be tested neat/raw or multiple straws of extended semen should be pooled for testing.

PCR testing vaginal fluid is not considered a useful option. Hazelton[15] showed that only 18% of cows with *M. bovis* mastitis tested positive to vaginal swab PCR. This test is therefore less sensitive than blood ELISA.

Testing embryos with PCR would be a novel approach. Testing an actual embryo would not convey the status of all embryos in the collection since *M. bovis* may not adhere to all zona pellucidas with the same affinity. However, the first fluids collected when the cow is flushed should contain a large number of cells, including uterine (epithelial) cells and dead sperm cells, on which *M. bovis* is known to adhere. Such a test would need to be validated, but it is believed that the results would reflect the status of the embryos that were collected from that flush.

A new technique of tonsillar crypt swabbing specifically for detection of asymptomatic animals has been developed as part of the New Zealand response. This technique is considered highly sensitive and not as limited by shedding like other common swab locations. Because tonsil swabs are a novel and intrusive technique requiring special training, it may pose difficulties in some situations.

7.3 Culture

M. bovis infections can be diagnosed by bacteriological culture, but it is difficult and time consuming. False-negative results can be common. Serological methods and molecular identification methods would be preferred. A PCR positive test should disqualify the donor or germplasm, even if subsequent culture was negative. Samples which are cultured may not be representative of the germplasm collected for export.

8 Limitations of scientific knowledge

Since the IHS was issued, there is new information supporting *M. bovis* transmission through artificial insemination, and further evidence for the efficacy of the CSS GTLS protocol (*M. bovis* inoculation up to 10^4 cfu/ml). As discussed in the RRA, there is also evidence of *M. bovis* developing antibiotic resistance. The organisms' resistance is thought to be multifactorial.

Mycoplasmas have been shown to form biofilms *in vivo*[16,17] and *M. bovis* persistence in the host is thought to be a function of the strain's ability to form biofilm in the host[18], but evidence is lacking[19]. Additionally, it is unknown if *M. bovis* biofilm is important in disease transmission. Although there is evidence of *M. bovis* contamination of semen from the reproductive tract, there is no evidence of *M. bovis* biofilm contamination in processed semen. Other types of bacterial biofilms have been found in human semen[20], and there is evidence that seminal fluid decreases bacterial surface adherence and promotes aggregation to form biofilm[21]. It is unknown if *M. bovis*

[15] Hazelton MS, Sheehy PA, Bosward KL, Parker AM, Morton JM, Dwyer CJ, Niven PG, House JK, "Short communication- Shedding of *Mycoplasma bovis* and antibody responses in cows recently diagnosed with clinical infection", 2018, J. Dairy Science 101:1 584–589.

[16] Simmons WL, Dybvig K, "Mycoplasma Biofilms Ex Vivo and In Vivo", FEMS Micorobiol Lett. 2009 Jun; 295 (1): 77-81. Link

[17] Raymond BBA, Jenkins C, Turnbull L, Whitchurch CB, Djordjevic SP, "Extracellular DNA release from the genome-reduced pathogen *Mycoplasma hyopneumoniae* is essential for biofilm formation on abiotic surfaces", Scientific Reports, 2018, 8(1), 17697. Link

[18] Bürki S, Frey J, Pilo P, "Virulence, persistence and dissemination of *Mycoplasma bovis*" 2015, vol 179, 15-22. Link

[19] McAuliffe L, Ellis RJ, Miles K, Ayling RD, Nicholas RAJ, "Biofilm formation by mycoplasma species and its role in environmental persistence and survival", Microbiology 2006, 152, 913-922. Link

[20] Swidsinski A, Dörffel Y, Loening-Baucke V, Mendling W, Verstraelen H, Dieterle S, Schilling J, "Desquamated epithelial cells covered with a polymicrobial biofilm typical for bacterial vaginosis are present in randomly selected cryopreserved donor semen", FEMS Immunology & Medical Microbiology, Volume 59, Issue 3, August 2010, Pages 399–404. Link

[21] Anderson MT, Byerly L, Apicella MA, Seifert HS, "Seminal Plasma Promotes Neisseria gonorrhoeae Aggregation and Biofilm Formation" J Bacteriol. 2016 Aug 15; 198(16): 2228–223. Link

behaves similarly to other bacteria in semen. It is also unknown how semen processing, freezing, and insemination would affect cells within the biofilm.

A study of *Mycoplasma* biofilm by McAuliffe[22] showed that *M. bovis* biofilms were not significantly more resistant to certain antibiotics during *in vitro* testing. About a decade later another *in vitro* study[23] demonstrated that another species of *Mycoplasma* biofilms are significantly (about 500x) more resistant to antimicrobials than planktonic cells. Both *in vitro* studies demonstrated that antibiotics inhibit polysaccharide film formation at lower concentration for planktonic compared to biofilm cells. A relatively higher concentration of antibiotics may be required to prevent biofilm expansion in semen, but perhaps only for certain strains. More *in vivo* research on biofilm formation in semen and the efficacy of antibiotics used in bovine semen on *M. bovis in* biofilm is warranted.

Due to concerns about antibiotic resistance, other ways to manage *M. bovis* should be explored. Finding therapies that penetrate or disrupt biofilms may be justified. For example, glycerol monolaurate (GML), was demonstrated to be safe on mucosa and very effective on *Mycoplasma hominis* and other types of bacteria in biofilms by Schlievert and Peterson[24]. Solubilisation of GML in non-aqueous vehicles is standard, but these may be more toxic to sperm cells. The bactericidal concentration of GML required for *M. hominis* was so low that it could be solubilised in an aqueous solution and Schlievert and Paterson showed that doing so improved its bactericidal effect.

How embryos become contaminated with *Mycoplasmas* is unknown. It is known that *M. bovis* can adhere to embryos and is not completely removed by washing, however washing could be expected to remove aggregates of *M. bovis* and biofilm if present. It is unknown if biofilm, aggregates, or cells adhered to embryos would vary in susceptibility to antibiotics.

As the body of evidence about *M. bovis* resistance and transmission from the bovine reproductive tract grows, studies to confirm efficacy of antibiotics in semen as well as biofilm therapies may become important.

This discussion document and the RRAs on *M. bovis* in semen and embryos reveal knowledge gaps specifically related to the:

a) transmission of *M. bovis* from infected germplasm (unprocessed versus internationally traded)
b) infective dose (in general and specifically from artificial reproduction)
c) efficacy of CSS protocols, specifically the GTLS combination, for semen and IETS recommendations for embryos
d) role of temperature and the cryoprotectants used in the CSS protocol
e) nature of *M. bovis* colonisation of the reproductive tract
f) sites of colonisation and characteristics of semen contamination
g) efficacy of current OIE recommended antibiotic combinations
h) novel therapies to prevent or eliminate *M. bovis* adherence to cells, and aggregate and biofilm formation
i) existing diagnostics

[22] McAuliffe L, Ellis RJ, Miles K, Ayling RD, Nicholas RAJ, "Biofilm formation by mycoplasma species and its role in environmental persistence and survival", Microbiology 2006, 152, 913-922. Link

[23] Tassew DD, Mechesso AF, Park N, Song J, Shur J, Park S, "Biofilm formation and determination of minimum biofilm eradication concentration of antibiotics in *Mycoplasma hyopneumoniae"* Journal of veterinary Medical Science, 2017, 79: 1716. Link

[24] Schlievert PM and Peterson ML, "Glycerol Monolaurate Antibacterial Activity in Broth and Biofilm Cultures", 2012, 7(7).

9 Summary

The risk of *M. bovis* entry with semen and embryos is assessed to be low and very low respectively. The risk of transmission from processed germplasm is assessed to be very low. There is no evidence that imported semen was the source of New Zealand's outbreak in 2017.

Compared to *M. bovis* transmission studies that existed when the import health standards (IHSs) were written in 2010, there is now one peer reviewed study of *M. bovis* transmission from processed semen, evidence of increasing antibiotic resistance, and one study indicating ineffective antibiotic control when extender and antibiotics are premixed.

MPI and New Zealand industry are committed to attempting eradication. In terms of germplasm, domestic suppliers are undertaking surveillance, and movement/use restrictions are put in place on germplasm when Notice of Direction restrictions are issued on properties.

If additional *M. bovis* requirements were added to the IHSs for bovine semen and embryos, it is possible that some countries/exporters would not meet them, and New Zealand's access to exotic genetics could become limited.

10 Risk management options

Under each heading below is a list of risk management options. The first option (highlighted in grey) is the current IHS requirement. The additional options are scientifically justified ways risk management could be increased. How much each requirement would increase risk management is unknown; it may only be marginal since the risk is already low.

10.1 Germplasm processing

(1) Collection and processing aligned with the recommendations of the OIE *Code* (including the three antibiotic combinations) and semen was held at a temperature of at least 5°C for a period of not less than 45 minutes before freezing; or

(2) Collection and processing of germplasm was in accordance with the recommendations of the OIE *Code*, except the following antibiotic combination must be used at the specified final dose per mL of embryos in media and extended semen:

a) Gentamicin (500 μg), tylosin (100 μg), lincomycin–spectinomycin (300/600 μg) (GTLS); or

i) Another MPI approved antibiotic combination; and

b) Antibiotics must be prepared and stored as separate stock solutions as described by the manufacturer to maintain potency; and
c) Antibiotics must be added to media/extender on the day of processing; and
d) The semen and embryos must remain in the antibiotic solution at the recommended concentration for a minimum of 2 hours at no less than 5°C before being frozen in the antibiotic solution.

10.2 Semen

(1) The donor has never tested positive for *M. bovis; or*

(2) The donor has always resided in a country free from *M. bovis;* or

(3) The donor has always resided in a *M. bovis* free herd in a country with a control programme that has been recognised by MPI; or

(4) An aliquot from 5 pooled straws of extended semen or an aliquot of neat /undiluted semen from each semen collection was tested with a validated, MPI approved PCR test for *M. bovis* with negative results.

10.3 Embryos

(1) The donor has never tested positive for *M. bovis; or*

(2) The donor has always resided in a country free from *M. bovis;* or

(3) The donor has always resided in a *M. bovis* free herd in a country with a control programme that has been recognised by MPI; or

(4) Every donor in the collection herd previously resided in no more than one other herd; and

- a) Every donor's herd of origin had been subjected to monthly MPI approved bulk milk tank and hospital/discard milk ELISA tests for *M. bovis* for the three months immediately prior to the donor joining the collection herd, with only negative results; and
- b) During the three months immediately prior to the donor joining the collection herd, only animals of equivalent *M. bovis* tested health status entered the herd of origin; and
- c) Before and after embryo collection and at least every month throughout residence:
  - i) All lactating cows in the collection herd were included in an MPI approved bulk milk tank ELISA test for *M. bovis,* with negative results; and
  - ii) Dry (non-lactating) cows were tested serologically with an MPI approved ELISA test for *M. bovis*, with negative results; and
- d) All semen used in the collection herd during the donor's residence was tested with an MPI approved PCR test for M bovis with negative results on a sample of 5 pooled straws of extended semen or an aliquot of neat /undiluted semen; or

(5) Every donor in the collection herd previously resided in no more than one other herd; and

- a) Every donor's herd of origin has ELISA tested every animal in the herd for *M. bovis* upon entry and at least annually for the past 2 years, with only negative results; and
- b) Every case of calf pneumonia has been investigated for *M. bovis,* and there have been no *M. bovis* positives for at least the past 3 years; and
- c) The donor had a vaginal fluid PCR test for *M. bovis* in the month before collection for New Zealand*,* with negative results; and
- d) All semen used in the collection herd during the donor's residence was tested with an MPI approved PCR test for *M. bovis* with negative results on a sample of 5 pooled straws of extended semen or an aliquot of neat /undiluted semen; or

(6) The embryo donor was tested using an MPI approved serum ELISA test for *M. bovis*, with negative results; and

- a) The embryos were subjected to a higher concentration of tylosin (200 µg/mL) and incubated at 37°C in the antibiotic treatment for a minimum of 4 hours after being washed 10 times; or

(7) The first flush media used in the embryo collection was tested with an MPI approved PCR test for *M. bovis* with negative results.

Appendix 1:

The effect of different extender protocols on viability of *Mycoplasma bovis* in bovine semen

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Backgound

Mycoplasma bovis (*Mb*) is a major bovine pathogen causing substantial economic losses and has a debilitating effect on animal welfare. *Mb* causes a variety of diseases including mastitis, pneumonia, arthritis, otitis media and genital infections. Antibiotic resistance is increasingly reported in *Mb* in Europe and efforts to develop efficacious vaccines have not been successful. Therefore it is of paramount importance to prevent the introduction of the agent into naïve herds.

We recently showed contaminated semen used in artificial insemination (AI) to be source of *Mb* infection in naïve dairy herds (Haapala et al., 2018). Several types of antibiotics have been added to seminal extenders before freezing to control bacterial contamination. GTLS mixture (gentamycin-tylosin-lincomycin-spectinomycin) is most widely used in bovine semen production (Shin et al., 1988), although it's ability to control *Mb* has been questioned (Visser et al., 1999). Recently also a fluoroquinole, ofloxacin, was shown to be effective in protecting semen from bacteria (Gloria et al., 2014).

Aim of the Study

• to evaluate the effect of different antibiotic/extender protocols on viability of *Mb* in bovine semen after freezing

Materials and Methods

Six extender/antibiotic protocols were compared:
1)ready to use extender containing GTLS
2)GTLS supplement added prior to use into the extender,
3)GTLS supplement added to semen before extending (USA requirements)
4)ofloxacin 100 µg/ml added prior to use into the extender
5)ofloxacin 400 µg/ml added prior to use into the extender
6)extender without antibiotics

Semen was collected from three bulls in AI center and processed using the same procedures that are used to produce AI straws for international trade. Two *Mb* strains (a field isolate from bull semen and ATCC 27368) and two concentrations (10^6 and 10^3 cfu/ml final concentration *Mb* in diluted semen) were used. Friis (F) medium (Bölske 1988) was used as control. The temperature of each extended semen lot with or without antibiotics was allowed to stabilize to room temperature (approximately one hour) after which automatic semen straw filling and sealing machine was used. The straws were stored in liquid nitrogen for 5 weeks before analysis. Fresh semen before extending was analyzed for *Mb* using culture.

Mb viability testing after freezing:
Three pooled samples of each trial lot were examined. Each pooled sample consisted of 6 straws. Ten-fold dilutions up to 10^{-5} were made into F broth in tightly closed tubes. Broth cultures were incubated at 37 °C for 14 days. The growth and colour change were monitored every other day. *Mb* was identified using *oppD* real-time PCR (Sachse et al., 2010).

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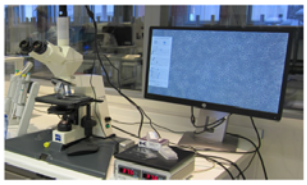
Results

- No *Mb* was detected in the semen before spiking
- *Mb* viability in different lots after freezing are shown in table below:

Antibiotic/extender	Strain/conc.	Culture A	Culture B	Culture C
GTLS ready to use	F broth	neg	neg	neg
GTLS ready to use	ATCC 10^3	pos	pos	pos
GTLS ready to use	ATCC 10^6	pos	pos	pos
GTLS ready to use	Bull 10^3	neg	neg	pos
GTLS ready to use	Bull 10^6	pos	pos	pos
GTLS added prior to use	F broth	neg	neg	neg
GTLS added prior to use	ATCC 10^3	neg	neg	neg
GTLS added prior to use	ATCC 10^6	pos	pos	pos
GTLS added prior to use	Bull 10^3	neg	neg	neg
GTLS added prior to use	Bull 10^6	pos	pos	pos
GTLS USA protocol	F broth	neg	neg	neg
GTLS USA protocol	ATCC 10^3	neg	pos	neg
GTLS USA protocol	ATCC 10^6	pos	pos	pos
GTLS USA protocol	Bull 10^3	neg	neg	neg
GTLS USA protocol	Bull 10^6	pos	pos	pos
Ofloxacin 100 µg	F broth	neg	neg	neg
Ofloxacin 100 µg	ATCC 10^3	pos	pos	pos
Ofloxacin 100 µg	ATCC 10^6	pos	pos	pos
Ofloxacin 100 µg	Bull 10^3	neg	neg	neg
Ofloxacin 100 µg	Bull 10^6	pos	pos	pos
Ofloxacin 400 µg	F broth	neg	neg	neg
Ofloxacin 400 µg	ATCC 10^3	pos	neg	pos
Ofloxacin 400 µg	ATCC 10^6	pos	pos	pos
Ofloxacin 400 µg	Bull 10^3	neg	neg	neg
Ofloxacin 400 µg	Bull 10^6	pos	pos	pos
No antibiotics	F broth	neg	neg	neg
No antibiotics	ATCC 10^3	pos	pos	pos
No antibiotics	ATCC 10^6	pos	pos	pos
No antibiotics	Bull 10^3	pos	pos	pos
No antibiotics	Bull 10^6	pos	pos	pos



Conclusions

None of the protocols had any effect on viability of *Mb* at 10^6 cfu/ml. Extender without antibiotics and freezing did not eliminate *Mb*. At lower concentration the ATCC strain seemed to be more resistant, whereas all protocols except GTLS ready to use and extender without antibiotics inhibited the growth of bull strain.

None of the protocols was superior and differences between strains occurred. Therefore it is challenging to use antibiotics in bovine semen production to control *Mb*. Further work in this topic is needed.

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