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A REDUCTION IN THE DURATION OF INFECTION WITH *TRITRICHOMONAS FOETUS* FOLLOWING VACCINATION IN HEIFERS AND THE FAILURE TO DEMONSTRATE A CURATIVE EFFECT IN INFECTED BULLS

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ABSTRACT

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Seven batches of 25 % water-phase, oil-in-water vaccine were prepared from whole cultures of *Tritrichomonas foetus*. Two inoculations were given, spaced 6 weeks apart, to virgin heifers and infected bulls.

A significant reduction ($P < 0,01$) in the duration of infection in vaccinated heifers was seen when they were challenged by being bred to a bull infected with the same isolate as that contained in the vaccine. Only 1/12 vaccinated heifers were pregnant 4,5 months after the end of the breeding season compared to 2/12 in the control group. The vaccine, therefore, has no practical advantage.

Vaccine was supplied to 2 724 bulls on properties where the infection was present. From these bulls, 110 reliable results were obtained, where bulls had been infected, been inoculated and tested 1 month later. No curative effect was demonstrable with 69/110 (62,7 %) bulls, remaining infected after the course of inoculations. There was also no difference between vaccine batches or between bulls of different ages. Further work on improving the vaccine is indicated.

Three media suitable for the culture of *T. foetus* are described in detail.

INTRODUCTION

Trichomoniasis, which is characterized by lowered fertility in cattle with few early abortions (shorter than 5 months gestation) actually being observed, is world-wide in distribution and occurs where natural service is practised (Abbitt, 1986; Skirrow & BonDurant, 1988). No true surveys of the incidence of the disease have been published as all have a bias of either coming from properties with low fertility or from old or slaughter bulls. Nevertheless, BonDurant (1985) mentions infection rates in bulls of 7–8 %. In the Diagnostic Laboratory of the Reproduction Section of the Veterinary Resarch Institute (VRI). Onderstepoort, 100/760 (13 %) bulls and 26/84 (31 %) herds were found to be infected with *Tritrichomonas foetus* (unpublished annual report, 1989/90) but many of these specimens came from properties experiencing poor fertility. The economical loss caused by the disease was estimated as $800 per infected bull in beef herds in the USA in 1958, $7 m per annum for the state of Oklahoma in 1979 and $665 per infected dairy cow in California in 1986 (Skirrow & BonDurant, 1988). Losses of similar economic significance can be expected in infected herds in the RSA.

A vaccine to control the ravishes of the disease would be of great value. Morgan (1947) showed that 16 injections of *T. foetus* antigen gave protection, but this would not be practicable. More recently, oil-adjuvanted vaccines have given promising results as curative agents in bulls (Clark, Dufty & Parsonson, 1983; Clark, Emery & Dufty, 1984; Campero, Hirst, Ladds, Vaughan, Emery & Watson, 1990) and were found to reduce the duration of infection in females (Kvasnicka, Taylor, Huang, Hanks, Tronstad, Bosomworth & Hall, 1989).

In the search for a viable solution to the problem of trichomoniasis, an experimental, oil-adjuvanted *T. foetus* vaccine was produced and tested for its prophylactic effect in heifers and curative effect in bulls.

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MATERIALS AND METHODS

Experimental animals

Twenty-four virgin heifers of Brahman, Hereford and Hereford cross breeds, with a body mass of between 255 and 426 kg at the time of the first vaccination and between 308 and 476 kg at the time of breeding 2,5 months later, were used to test the prophylactic action of the vaccine. Before vaccination, rectal palpation of the ovaries was done and recorded as either active or non-active. The heifers were selected at this time into matching pairs according to breed, mass and the presence or absence of active ovaries. At the time of breeding 2 heifers still had palpably inactive ovaries, but it was decided to include them in the trial nevertheless.

Except for inoculation with *Brucella abortus* strain 19 vaccine between the ages of 3 and 8 months, the heifers were not inoculated with any other vaccine. The heifers tested negative for trichomoniasis and campylobacteriosis on 2 vaginal mucus specimens collected 6 weeks and 4 days before joining with a *Tritrichomonas*-positive bull on 19 September, 1989. They were tested serologically for brucellosis, leptospirosis, Q fever, infectious bovine rhinotracheitis (IBR), bovine viral diarrhoea, bluetongue, Rift Valley fever (RVF), Wesselsbron disease (WD), Akabane virus and chlamydiosis at 2-monthly intervals before, during and at the end of the trial, which was terminated 4,5 months after the removal of the bull. Seroconversion in any of these diseases was accepted when the titre changed from negative to 64 or higher or, in the case of initial titres, if it increased fourfold.

The bull used to join with the heifers was a 5-year-old grade Friesland that had been infected intrapreputially with a culture of a field isolate (1067/8) of *T. foetus* at the age of 3 years and had remained permanently infected. He was negative for campylobacteriosis on more than 3 separate preputial washings spaced more than 1 week apart. His semen was of satisfactory quality as regards density, motility and the limits of abnormalities. This was the first time that he had been used for breeding.

TABLE 1 Media used for the successful isolation of *T. foetus*

Ingredient	Modified Plastridge	Modified Stenton	Oxoid CM 161
Trichomonas medium CM 161[a]	—	—	37,5 g
Nutrient broth[b]	16 g	16 g	—
Ascorbic acid[b]	—	0,75 g	—
Agar[c]	0,7 g	1 g	—
Sterile distilled water[d]	750 mℓ	900 mℓ	900 mℓ
D (+) Glucose[e]	10 g	—	—
Sterile dist. water for glucose[f]	50 mℓ	—	—
Sterile horse or cattle serum[g]	200 mℓ	100 mℓ	100 mℓ

[a] Oxoid Ltd., Basingstoke, Hampshire, England
[b] Biolab Chemicals, 2 Bernard St., Colbyn 0083, Pretoria, South Africa
[c] Biolab Diagnostics, P.O. Box 1998, Halfway House 1685, South Africa
[d] The above ingredients are dissolved in the stated quantity of water and autoclaved at 121 °C for 15 min, then cooled to 50 °C before the other ingredients are added
[e] E. Merck, Darmstadt, West Germany
[f] The glucose is dissolved in this amount of water and autoclaved at 109 °C for 20 min, cooled to 50 °C and then added to the rest of the medium
[g] The sterile horse or cattle serum is inactivated at 60 °C for 30 min and then added to the rest of the medium
1. The media are dispensed in 12 mℓ aliquots in 20 mℓ screwcapped McCartney bottles
2. Sterile liquid paraffin (0,5 mℓ) is added to each bottle of modified Stenton's medium to create an anaerobic condition in the medium
3. The media may be stored at 4 °C for up to 1 month before use
4. On the day that the media are to be inoculated an antibiotic mixture, as outlined below, is added to each 12 mℓ of medium to control bacterial and fungal contaminants

Antibiotics	
Sodium benzylpenicillin[h] (1 000 000 i.u.)	0,6 g
Streptomycin sulphate[i]	1,0 g
Amphotericin B[j]	5,0 mg
Sterile distilled water[k]	100,0 mℓ

[h] Novopen, Novo Industries, P.O. Box 783155, Sandton 2146, South Africa
[i] Novo-strep, Novo Industries, as above
[j] Fungizone for tissue culture, E. R. Squibb & Sons Inc., Princeton, NJ 08540, USA
[k] After dissolving the antibiotics in the water, 1 mℓ of the solution is added to each 12 mℓ aliquot of medium, giving a final concentration per mℓ of:
769 i.u. or 462 µg penicillin
769 µg streptomycin sulphate
3,8 µg amphotericin B

5. Inoculated media are incubated at 32–37 °C and examined at 48, 96 and 144 h before being discarded as negative. A drop from the bottom of the culture is taken as more trichomonads congregate at this level

Vaccine was distributed under field conditions to farmers for the inoculation of 2 724 bulls on 135 properties where the infection had been diagnosed. Reliable data were obtained from 110 bulls which had been tested positive for *T. foetus,* had subsequently been vaccinated and were then retested 1–3 times. Those that were positive on 1 or more occasions on retest were accepted as being still infected. The bulls were of a variety of breeds and were between 3 and 10 years old.

Specimens and methods for positive identification of T. foetus

Vaginal mucus was collected by passing a plastic, artificial insemination pipette through the vulva to the fornix of the vagina and applying suction with a 20 mℓ syringe. If sufficient mucus, i.e. more than 2,5–5 cm in the pipette, was obtained, it was removed by flushing with 5 mℓ of phosphate-buffered saline (PBS) of pH 7,2 into sterile 15 mℓ screwcap bottles. These were placed in a closed polystyrene holder and kept at 4 °C until further processing within 4 h in the laboratory. If insufficient mucus was obtained by this method, 5 mℓ of PBS was introduced by the same pipette into the fornix of the vagina, recovered by suction, and then handled like the other specimens. Separate sterile pipettes and syringes were used for each heifer. Vaginal mucus was collected from the heifers on a weekly basis, commencing 1 week after the introduction of the bull and continuing for 27 weeks. During the 14th week (Christmas), no specimens were taken (Tables 2 & 3). At slaughter, done during the 3 weeks after termination of the trial (at the 27th week), specimens were taken with sterile precautions from the uterine content of all animals and from the foetal abomasal content, if pregnant. In the cases of abortion and in the case of badly mutilated foetus, foetal abomasal content or deep swabs of the external auditory meatus were taken for wet mount examination and culture for *T. foetus*. The cows were slaughtered the next day and uterine content similarly examined.

Preputial washes from bulls in the field were done by veterinarians, advised to follow the method described by Pefanis, Herr, Venter, Kruger, Queiroga & Amaral (1988), using 50 mℓ PBS with preputial massage of 100 times and specimens delivered to the laboratory within 8 h.

In the laboratory, preputial washes were centrifuged and processed as described by Pefanis *et al.*, (1988). From both the reconstituted sediment of centrifuged preputial washes and directly from the vaginal mucus specimens, a wet mount of 1 drop under a coverslip was examined at 100 × by phase-contrast microscopy for the characteristic jerky motion of the *T. foetus* organism. This was confirmed at 400 × by visualizing the 3 anterior flagella and the undulating membrane. A further 4 drops from each specimen were inoculated in 12 mℓ each of a modified Stenton's medium (Lowe, 1978) and either a modified Plastridge's medium (Plastridge, 1943) or a commercial *Trichomonas* medium (Oxoid) all prepared as described in Table 1. The media were incubated at 32 °C and a wet mount examined as above at 96 and 144 h before being discarded as negative.

Vaccine and vaccination

Vaccine was produced by culturing a preputial wash specimen from the same bull as was to be used for breeding. It was cultured initially in 100 mℓ of a modified Plastridge's medium (Plastridge, 1943) (Table 1) with selective antibiotics added as recom-

TABLE 2 Duration of infection with *Tritrichomonas foetus* in 12 unvaccinated heifers

Weeks post-introduction of infected bull																										Total
0 1[a]	0 2	0 3	0 4	0 5	0 6	0 7	0 8[b]	0 9	1 0	1 1	1 2	1 3	1 4[c]	1 5	1 6	1 7	1 8	1 9	2 0	2 1	2 2	2 3	2 4	2 5	2 6	2 7[d]	
−	−	+	+	+	+	+	+	+	+	−	−	+		−	−	−	−	−	−	−	−	−	−	−	−	−	11[e]
−	−	+	+	+	+	+	+	−	−	+	+	+		−	−	−	−	−	−	−	−	−	−	−	−	−	11
+	+	+	+	+	+	+	+	+	+	+	+	+		−	−	−	−	−	−	−	−	−	−	−	−	−	13
−	−	+	+	+	+	+	+	+	+	+	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	9
−	−	−	−	+	+	+	+	+	−	−	+	+		−	+	+	+	+	−	−	−	−	−	−	−	−	15
−	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	−	+	+	−	25
−	−	−	+	+	+	+	+	−	+	+	+	+		+	−	−	+	−	−	−	−	−	−	−	−	−	15
+	+	+	+	−	−	−	+	+	+	+	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	11
−	−	−	+	+	+	+	+	+	−	−	−	−		−	+	+	−	−	−	−	−	−	−	−	−	−	14
−	−	+	+	+	+	+	+	+	+	−	−	−		−	+	+	+	−	−	−	−	+	−	−	A[f]		16
−	−	−	−	+	−	−	−	+	+	+	+	+		+	−	+	−	+	−	−	−	−	−	−	−	−	15
−	+	+	+	+	+	+	+	+	−	−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	8
																									Mean		14

[a] 1st week
[b] Bull removed on day 60
[c] Christmas, no tests done during 14th week
[d] 27th week
[e] Duration of infection including intervening weeks of negative findings except in animal that aborted where positve finding prior to abortion was ignored
[f] Aborted, *T. foetus*-positive, dam slaughtered next day

TABLE 3 Duration of infection with *Tritrichomonas foetus* in 12 vaccinated heifers

Weeks post-introduction of infected bull																										Total
0 1[a]	0 2	0 3	0 4	0 5	0 6	0 7	0 8[b]	0 9	1 0	1 1	1 2	1 3	1 4[c]	1 5	1 6	1 7	1 8	1 9	2 0	2 1	2 2	2 3	2 4	2 5	2 6	2 7[d]	
−	−	+	+	+	+	+	−	−	−	−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	5
−	−	+	+	+	+	+	+	−	−	−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	6
−	−	−	−	+	+	+	+	−	−	−	+	+		−	−	−	−	−	−	−	−	−	−	−	−	−	9[e]
−	−	−	+	+	+	+	+	−	−	−	−	−		−	−	−	−	−	−	+A[f]							5
−	−	+	+	+	−	−	−	−	−	−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	3
+	+	+	+	+	−	−	−	−	−	+	+	−		−	−	−	−	−	−	−	−	−	−	−	−	−	12
−	+	+	+	+	−	+	−	−	−	−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	6
−	+	+	+	+	−	+	+	+	−	−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	8
−	+	+	+	+	+	+	+	−	−	−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	7
+	+	+	+	+	+	−	−	+	+	+	+	−		−	−	−	−	−	−	−	−	−	−	−	−	−	12
−	−	+	+	+	+	−	−	−	−	−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	4
−	−	+	+	+	+	+	+	−	−	−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	6
																									Mean		7

[a] 1st week
[b] Bull removed on day 60
[c] Christmas, no test done during 14th week
[d] 27th week
[e] Duration of infection including intervening weeks of negative findings except in case of animal that aborted where positve finding prior to abortion was ignored
[f] Aborted, *T. foetus*-positive, dam slaughtered next day

mended by Reece, Dennett & Johnson (1983). For larger quantities of vaccine, a 10 % inoculum of a culture with 1×10^8 organisms per mℓ was used in the increased volume. The pH was controlled at 7,2. All media were incubated at 37 °C. When a growth of between 1×10^9 and 5×10^{10} organisms per mℓ was achieved, the culture was used as the water phase of 25 % in a water-in-oil emulsion. Seven batches of vaccine were made from cultures of the isolate 1067/8. The first 3 batches contained 1 ℓ culture each, the 4th 5 ℓ, the 5th and 6th 10 ℓ each and the 7th 300 ℓ. All batches were used in the field work on bulls and batch 6 for the 1st and 7 for the 2nd inoculation of the heifers.

The bulls were vaccinated twice with a dose of 10 mℓ given intramuscularly (i/m) and spaced 6 weeks apart. Preputial washes were taken 1 month or more after the 2nd inoculation. The 2nd of each selected pair of heifers was given two 5 mℓ doses i/m, 6 weeks apart, with the last inoculation being 1 month before the breeding season of 60 days duration. The other heifer in each pair was left unvaccinated, but given a placebo of 5 mℓ PBS i/m.

Pregnancies and abortions

Rectal palpation for pregnancy diagnosis was done 4,5 months after removal of the bull to ensure that no effects, however unlikely, resulting from this procedure could influence foetal survival. The correctness of the pregnancy diagnoses was checked when the trial was terminated by slaughter of all the remaining heifers 28 to 30 weeks after the introduction of the bull.

From the day of putting the heifers to the bull, both the heifers and the camp in which they were kept were closely monitored for any signs of abor-

tion. The age of each aborted foetus was taken as the time in weeks from the first recorded positive vaginal mucus test in that heifer (reflecting proof that she had been served) till the time of the abortion. Due consideration was given to the size and development of the foetus so that a subsequent service should not be erroneously taken for calculating the true age of the foetus. The same criteria were applied in judging the age of the foetus in the pregnant animals at slaughter.

RESULTS

No clinical consequences following inoculation were seen in the experimental heifers, and only a single field herd reported swelling at the site of injection.

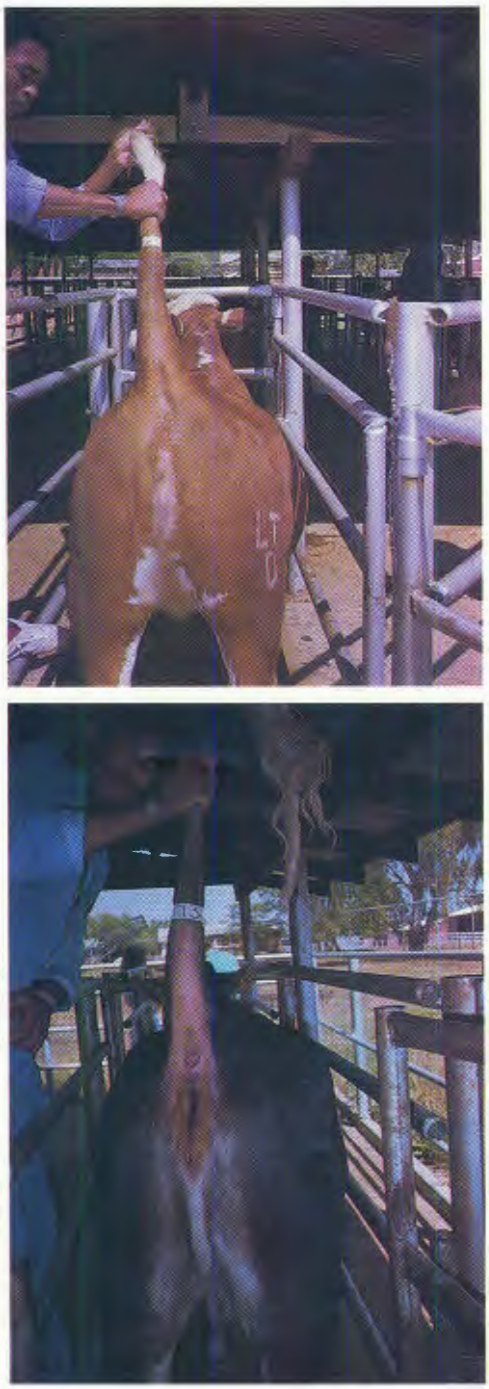

FIG. 1 The absence of pus on the tails of heifers infected with trichomoniasis for 5 (12) and 2 (13) weeks respectively

All the heifers became infected within 5 weeks of the introduction of the bull (Tables 2 & 3). The duration of the infection in the experimental, unvaccinated heifers averaged 14 weeks (Table 2) as opposed to 7 weeks in the vaccinated (Table 3). The t-test showed that this difference was significant ($P < 0,01$).

Although the vaginal mucosa was observed to be inflamed in all the heifers, no outward signs of infection were seen at any time and this is demonstrated by the absence of pus on the tails or pinbones of *Tritrichomonas*-infected heifers (Fig. 1). One vaccinated and 1 control heifer aborted *Tritrichomonas*-positive foetuses at 23 and 19 weeks gestation, respectively. The uterine content of these 2 aborters was also positive for *Tritrichomonas*. Two unvaccinated and 1 vaccinated heifers were diagnosed pregnant and confirmed at slaughter as being 23, 27 and 25 weeks pregnant, respectively. The pregnant and all the non-pregnant uteri and the 3 foetuses were *Tritrichomonas*-free at slaughter.

A good quality preputial wash, as illustrated in Fig. 2, was obtained in most cases, using the method described. Of the 110 previously infected bulls, 69 (62,7 %) were still infected more than 1 month after receiving 2 inoculations. In the 7 vaccine batches there were 3/7, 20/31, 13/28, 13/15, 8/9, 2/7 and 10/13 bulls that remained positive. There was no significant difference between the results from the various batches using the chi^2 test, although the batches with fewer than 10 animals may be non-representative. Of the 101 bulls for which ages were known, there were 5/12 positive among 3-year-olds, 16/30, 8/15 and 13/22 for 4-, 5- and 6-year-olds and 18/22 positive for bulls of 7 years or older. The chi^2 test showed no significant difference between these groups.

FIG. 2 The milkiness, opacity and froth seen with a good quality preputial wash compared with unused PBS

Two vaccinated heifers had titres to IBR > 64 and 1 titre to WD > 64 before the start of the trial and all these titres returned to negative during the experiment. One vaccinated and 1 unvaccinated heifer seroconverted to RVF during the trial. One vaccinated and 3 unvaccinated heifers seroconverted to WD while 1 vaccinated and 2 unvaccinated heifers seroconverted to IBR during the trial. All the above heifers were not pregnant at the termination of the experiment and none of them aborted. None of the heifers showed seroconversion from negative status to any of the other diseases which were serologically tested.

DISCUSSION

The seroconversions to RVF, IBR and WD were not regarded as having any effect on the trial as infertility and resorption are not reported with these diseases.

While the infected bull ran with both groups of heifers (60 days) there was the possibility that they could have been cleared of infection and re-infected. Nevertheless, the significantly shorter (7 weeks) duration of infection seen in the vaccinated heifers compares favourably with the 2 weeks' reduction reported for a vaccine used by Kvasnicka *et al.*, (1989) and with a reduction from a duration of infection of 13–28 weeks on primary challenge in unvaccinated stock, compared to 4 weeks with subsequent infection (Skirrow & BonDurant, 1990). There is, therefore, reason to believe that the vaccine has some immune effect, but the single pregnant animal among the vaccinated group compared to the 2/12 in the controls proves that this effect has no practical value. Further work on the vaccine and a closer look at the breeding criteria are indicated, as these results compare unfavourably with the reported 3/11 pregnancies with a different vaccine and where the bulls were kept with the females for 100 days, as opposed to our 60 days (Kvasnicka *et al.*, 1989). Although the bull used in this study could have been of low fertility, this is unlikely as the semen was of normal motility and density and his libido was proved by the infection of all the heifers within 6 weeks (Tables 1 & 2).

Success in the diagnosis of trichomoniasis, using vaginal mucus specimens, was satisfactory (Tables 2 & 3), but the individual heifers were not consistently positive. The reason for the negative findings during some weeks in a series of positive cases could be due to a lack of sensitivity in our test techniques. Even were this the sole cause, our test picked up infection in 85 % of cases. Another possible contributing factor could be some cyclical variation in numbers or viability of the organisms in the vagina. The varying patterns of intermittent negative findings do not, however, suggest that they are in any way related to the oestrus cycle. The success of diagnosis on vaginal mucus cannot be translated into an equal success under field conditions, where the time of infection is unknown. Twenty weeks after first exposure to infection, only 1/12 (8,3 %) control animals were still infected. The dissappearance of infection from the vagina for protracted periods in the 2 animals that aborted (Tables 2 & 3), with reappearance shortly before and at the time of abortion is noteworthy. It could be explained by postulating that the infection persisted in the uterus, cleared up in the vagina, was isolated in the uterus by the dense cervical plug during pregnancy and only reappeared when this plug softened prior to the abortion. This was the reason for excluding the positive vaginal mucus culture found in the 2 aborters, shortly before the abortions, as the duration of vaginal infection was the criterion used (Tables 2 & 3).

Contrary to the findings of other workers (Clark *et al.*, 1983; 1984; Campero *et al.*, 1990), we could not demonstrate a curative effect in *T. foetus*-positive bulls. The strain of *T. foetus* used and the vaccine formulation, especially the oil-adjuvant, may be playing a critical role in our lack of success. The conditions of field transport and unprofessional inoculation of the bulls may have played some role in the poor success of the vaccine but if it is to be commercialized, it must be successful under these conditions. Although self-cure is uncommon and therefore immunity from natural infection unlikely in the older bull, this may be due to the superficial location of the organism in the male, but vaccination may somehow contribute to the local presence of immunoglobulins in the preputium (Soto & Parma, 1989; Campero *et al.*, 1990). The phenomenon of vaccine protection only in younger bulls (Clark *et al.*, 1983) was likewise not seen in our work. The reports that a cell wall component used as antigen in vaccine gave better immunity than whole cell cultures used previously (Clark *et al.*, 1983; 1984; Campero *et al.*, 1990) is very interesting. Everything indicates that our vaccine needs refinement.

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