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PAPERS

AN APPROACH FOR THE FIT AND COMPARISON OF DIFFERENT BAYESIAN MODELS FOR THE VALIDATION OF DIAGNOSTIC TESTS

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SUMMARY

We describe a stepwise approach for the Bayesian validation of diagnostic tests in absence of a gold standard. This stepwise approach, based on the Deviance Information Criterion (DIC), allows us to compare the simplest model (conditional independence model) with gradually more complex models allowing for conditional covariance between the sensitivities and specificities of one or two pairs of tests. Data are from a survey for *Trypanosoma evansi* in dromedary camels in Niger. All 729 animals were tested with a parasite detection test and three antibody detection tests. The model which allows a conditional dependence between the indirect agglutination test and the ELISA appeared to be the better model. This can be expected biologically since these two tests are based on the same purified antigen.

INTRODUCTION

Bayesian inference using Monte Carlo Markov Chain (MCMC) techniques for the validation of diagnostic tests in absence of a gold standard gain more and more popularity. Most models however are still based on the assumption of conditional independence between the different tests and assessment of the goodness of fit of the models is rarely executed. Relaxing the conditional independence assumption requires more diagnostic tests in the experiment setup to avoid overparameterization or the availability of reliable prior information on one or more parameters. In addition it is essential to verify whether the new, expanded model is in fact a model that better explains the observed data.

We compared 14 models for the validation of four diagnostic tests for *Trypanosoma evansi* causing Surra in dromedary camels (a widespread parasitological disease responsible for important economical losses) starting with the simplest model under conditional independence and gradually increasing the complexity by including conditional covariances between test pairs. Model comparison was based on the DIC (Spiegelhalter *et al*., 2002) a Bayesian version of the Akaike Information Criterion (AIC, Atkinson, 1980). This stepwise approach of adding more parameters followed by model fit and comparison allowed the selection of the simplest model which explained the data the best.

MATERIALS AND METHODS

Sampling and diagnostic tests

Four diagnostic tests were conducted on all 729 samples obtained from a survey in Niger. A parasite detection test, the Mini Haematocrite Centrifugation Technique (MHCT), was conducted in the field. On the serum three serological tests (Verloo *et al*., 2000) were conducted. The CATT/*T.evansi* a direct agglutination test using freeze dried Coomassie stained whole trypanosomes of the RoTat 1.2 Variable Antigenic Type (VAT) (Bajyana Songa *et al*., 1988; Verloo *et al*. 2001); the LATEX/*T.evansi* an indirect agglutination test detecting agglutinating antibodies against the purified RoTat 1.2 Variable Surface Glycoprotein (VSG) bound to latex particles and an Enzyme Linked Immune Sorbant Assay (ELISA/*T.evansi*)) test detecting different antibodies against the RoTat 1.2 VSG.

Statistical models

As summarized in Table 1 a total of 14 latent class models (Walter et al., 1988) were fitted (multinomial likelihood). All priors were uninformative beta(1,1) distributions except for the priors on the conditional covariances which were uniform(0,1) with an upper limit min(se1,se2)-se1*se2 for those between two sensitivities and min(sp1,sp2)-sp1*sp2 for the covariance between two specificities (Vacek,

1985). The conditional covariance was constrained to be positive because negative dependence is biologically unlikely to exist (Vacek, 1985; Gardner *et al.*, 2000).

Posterior densities of all parameters were obtained by Gibbs sampling using WinBugs 1.4 software (Gilks *et al*.,1994). Three parallel sequences with different starting values were run for each model and convergence was monitored with the Gelman-Rubin statistic (Gelman *et al*.,1995). Once this statistic converged to 1 for all observed parameters (including the deviance), the burn in period was determined. After burn in all models ran for another 10,000 iterations. Goodness of fit of the models was assessed by visually inspecting the expected and observed data and by calculating posterior predictive p values (Bayes p) (Gelman *et al*.,1995) which is defined as the probability that the replicated data could be more extreme than the observed data, measured (in this case) with the $\chi 2$ discrepancy quantity (Splus, Combinat library). Bayes p values with extreme tail-area values correspond to major failures of the model.

Subsequent models were compared by monitoring the DIC after convergence of the deviance (a lower DIC value corresponds with a "better" model). The DIC is composed of a Bayesian measure for fit or adequacy (the posterior mean deviance) penalized with an additional complexity term pD being the difference between the posterior mean of the deviance and the deviance at the posterior mean of the parameters. We refer to Spiegelhalter *et al*. (2002) for more details on the DIC. All statistics of the posterior distributions (mean, 2.5^{th}, 50^{th} and 97.5^{th} percentiles) were rounded according to their Monte Carlo error.

RESULTS

As summarized in table 1 all models fitted the data according to the posterior predictive p values which showed no extreme values. However it was striking that models with Bayes p values leading away from 0.5 showed increasingly more discrepancies between the observed values and the distribution of the expected values (mostly in cells with a low or zero number of observations, graphs not shown). Note that for the model 5,6 and 9,10 we did not calculate a Bayes p value but as they were simplifications of the models 4 and 8 and the deviance was similar to those models the fit was also expected to be adequate. According to the DIC, model 8, which allows the LATEX and ELISA test to be conditional dependent for both sensitivity and specificity was found the better model followed close by model 4. Simplification of that model by adding the covariance between sensitivities alone (model 9) or specificities alone (model 10) did not improve the DIC (worse deviance and only a slight better model complexity score pD). Model 12 was obtained by combining models 4 and 8. Not surprisingly this model scored a very good DIC but it was not superior to model 8. The conditional independent model (1) staid a better candidate than lot of other models for which a covariance was placed between the wrong pairs of tests.

Summary statistics of the posterior distributions of the parameters in model 8 are shown in table 2.

DISCUSSION

We agree with Box (1976) saying that that all statistical models are wrong but some are useful. Therefore model fitting, comparison and selection is crucial to statistical analysis but as stated by Spiegelhalter et al. (2002) an overformal approach to model 'selection' is inappropriate since so many other features of a model, e.g. the robustness of its conclusions and its inherent plausibility, should be taken into account before using it as a basis for reporting inferences. We believe that in this case the better model (8) is also the biologically plausible model leading to biologically acceptable parameter estimates (data not shown) but of course there are other (non tested) models and other ways to model the conditional dependencies of diagnostic tests.

For this paper we did not try to model conditional dependencies between three or more tests. This because of the inherent difficulty in interpreting those interactions and the necessity on inclusion of prior information to fight the overparametrisation. Next to this is the conditional covariance as defined by Vacek (1985) not straightforward for modelling conditional dependencies between three or more tests and alternative parametrisations have to be used. Modelling all possible interactions between the tests however is statistical utopia. There is even no setup possible for such an analysis as adding diagnostic tests to the setup increases exponentially the lack of degrees of freedom.

Remarks can be made on the fact why these models where not worked out in a frequentist framework since they are all identifiable models and we did not gave any shape to the prior distributions of the parameters. We are however convinced that the flexibility and the ease of simulation from the posterior distributions (leading to distributions of other parameters of interest as predictive values, diagnostic accuracy parameters of test combinations, sample size calculations, etc…) is a major advantage of MCMC next to the

fact that normal distribution approximations, in the frequentist framework commonly employed to derive confidence intervals around unknown parameters from estimated standard errors, are not required.

A lot of discussion is going on the use of the DIC as a Bayesian model comparison tool. One of the aspects is the pD as a measure for model complexity while in other information criteria like the widely used AIC the numbers of parameters to be estimated are a measure for model complexity. Using the pD however will allow the model complexity to be related to the data and the prior information put in the model which is very reasonable in the Bayesian context (e.g. penalization for adding 1 conditional covariance between two test sensitivities is restricted because of the constraint put on the parameter).

Table 1. Overview Bayes p values, number of parameters to be estimated, deviance, pD (model complexity) and DIC for the different models

Nr	Covariance between	Bayes p	Param	Deviance	pD	DIC
	NONE					
1*	(CI model)	0.36	9	66.396	7.419	73.815
	ONE PAIR OF TESTS					
2	CATT-LATEX	0.36	11	66.287	7.668	73.955
3	CATT-ELISA	0.26	11	68.469	7.803	76.272
4*	MHCT-CATT	0.52	11	64.039	8.537	72.576
5	Se		10	66.108	7.768	73.876
6*	Sp		10	64.251	8.150	72.402
7	MHCT-LATEX	0.27	11	67.901	7.751	75.652
8*	LATEX-ELISA	0.57	11	63.398	8.219	71.617
9	Se		10	65.543	8.104	73.647
10	Sp		10	67.746	7.886	75.632
11	MHCT-ELISA	0.32	11	66.914	8.496	75.411
	TWO PAIRS OF TESTS					
12*	MHCT-CATT & LATEX-ELISA	0.51	13	63.779	8.556	72.336
13	ELISA-MHCT & CATT-LATEX	0.31	13	67.346	8.117	75.463
14	MHCT-LATEX & ELISA-CATT	0.18	13	70.114	8.072	78.187

Table 2. Summary statistics posterior distributions of model 8

Parameter	Mean	2.5^{th} perc	50^{th} perc	97.5^{th} perc
Prevalence	0.5615	0.5233	0.5615	0.5987
Se MHCT	0.2548	0.2135	0.2545	0.2979
Se CATT	0.9756	0.9529	0.9762	0.9941
Se LATEX	0.9667	0.9433	0.9675	0.9854
Se ELISA	0.7856	0.744	0.7859	0.8248
Sp MHCT	0.9904	0.977	0.9914	0.9981
Sp CATT	0.9827	0.9577	0.9841	0.999
Sp LATEX	0.9833	0.9554	0.9855	0.9993
Sp ELISA	0.9798	0.9528	0.9817	0.9965

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ASSESSMENT OF THE POST TEST PROBABILITIES OF MULTIPLE TESTS IN ABSENCE OF A GOLD STANDARD IN ORDER TO SELECT THE APPROPRIATE TEST COMBINATIONS AND DECISION RULES

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SUMMARY

We describe the Bayesian validation of four antibody detection tests in absence of a gold standard. The aim of the study was to choose the best test combination to assess seronegativity of Belgian cattle against Bovine herpes virus 1. Four candidate ELISA tests were selected and executed on 920 field sera. Sensitivity, specificity and predictive values of all tests and different test combinations were assessed. Based on these results the best test combination and decision rule was chosen.

INTRODUCTION

EU directives for import and export of cattle are based on the fact that animals should be seronegative against Bovine herpes virus 1. Several antibody ELISAs are available but discrepancy between test results is frequent and questions raise on how to intepret these results and on which ELISAs to use in a sequential multiple test set up.

The Belgian reference lab selected two candidate blocking ELISAs and two indirect ELISAs and decided, in order to avoid dependence conditional on the seropositive status that multiple tests should be based on one blocking ELISA and one indirect ELISA. Guiding the decision on which blocking and which indirect ELISAs to choose and how to interpret the test results was the primary target of this study.

We used Bayesian inference by Monte Carlo Markov Chain (MCMC) techniques for the validation of the four candidate antibody ELISAs in absence of a gold standard. Pair wise dependence on the seropositive status was modelled and different biologically plausible models were fitted and compared. Tests an test combinations were evaluated according to their diagnostic accuracy (sensitivity and specificity) and their post test probabilities (predictive values).

MATERIALS AND METHODS

A total of 920 field sera were all, within the same laboratory, analyzed with four antibody detection ELISAs (2 glycoprotein B blocking ELISAs (T1 and T2) and 2 indirect ELISAs (T3 and T4)).

The Bayesian statistical approach and the model fitting and comparison are described in detail by Verloo & Büscher (2004). We compared different models using the deviance information criterion (DIC) (Spiegelhalter *et al.*, 2002) starting with the simplest model under conditional independence and gradually increased the complexity by including conditional covariances between test pairs. All priors were uninformative beta(1,1) distributions except for the priors on the conditional covariances which were uniform(0,1) with an upper limit min(se1,se2)-se1*se2 for those between two sensitivities and min(sp1,sp2)-sp1*sp2 for the covariance between two specificities (Vacek, 1985).

For different test combinations the sensitivity and specificity of a parallel (OR, believe the positive) interpretation scheme and a serial (AND, believe the negative) was calculated and corrected for conditional dependence according to Gardner (2000). We used the notations sep_{T1T2}

(spp_{T1T2}) for the sensitivity (specificity) of a parallel, and ses_{T1T2} (sps_{T1T2}) for the sensitivity (specificity) of a serial interpretation scheme of, in this case, T1 and T2.

Post test probabilities (PPV and NPV) were calculated using Bayes theorem. Inclusion of conditional covariances in the post test probabilities of multiple tests was as described by Gardner (2000). We used the notation $PPVpar_{T1T2}$ for the positive predictive value of a parallel interpretation scheme and $NPVser_{T1T2}$ which the negative predictive value of a serial interpretation scheme. Logically, the negative predictive value of a parallel interpretation scheme is $NPVpar_{T1T2}$ = 1-Pr(D+ | T1-,T2-) while the positive predictive value of a serial interpretation scheme $PPVser_{T1T2}$ = Pr(D+ | T1+,T2+).

When comparing tests or test combinations at the level of the diagnostic accuracy (se and sp) or at the level of the post test probabilities (PPV and NPV) we used se+sp as an overall measure of accuracy and PPV+NPV as a measure for post test knowledge.

Comparison of posterior distributions or linear combinations of posterior densities was done by random drawings from these distributions. To determine the confidence (a value between 0 and 1) that one parameter was higher than another parameter we calculated the proportion of draws which fulfilled that criterion.

RESULTS

According to the DIC the model which allowed a conditional dependence between the sensitivities and specificities of the two indirect ELISAs and two blocking ELISAs respectively, appeared to be the simplest model which explained the data the best (DIC=58.25 compared to a DIC of 150,73 for the conditional independent model; no further details on model comparison shown). Table 1 summarizes the posterior densities of the parameters of this model.

Comparison of the se+sp of the two blocking ELISAs and the two indirect ELISAs resulted in a 0.99 confidence that test 1 was the better one of the two blockings and a 0.64 confidence that T4 was the better one of the two indirect ELISAs. This was confirmed by the post test probabilities where the Pr(PPV1+NPV1>PPV2+NPV2) results in a 0.905 confidence and Pr(PPV4+NPV4>PPV3+NPV3) = 0.89

For above reasons it was chosen to continue the analysis of the multiple test setups with T1 as the preferred blocking ELISA and T3 or T4 as the indirect ELISAs.
When comparing Pr(D+ | T1+,T3+) and Pr(D+ | T1+,T4+) there is a 0.804 confidence in having a higher post test probability for the T1T4 combination while for the Pr(D- | T1-,T3-) and Pr(D- | T1-,T4-) no obvious difference can be found (0.523 confidence that the T1T4 combination is higher).

In addition, for a serial interpretation scheme the Pr(ses13+sps13<ses14+sps14)= 0.655 while for the PPVser + NPVser this is approximately 0.5. For a parallel interpretation scheme the Pr (sep13+spp13<sep14+spp14) ≈ 0.5 while for the sum of the PPVpar and NPVpar there is a 0.973 confidence that the T1T4 combination is the better one. This is completely caused by the difference in PPVpar although the difference is minimal (mean=median = 0.01 with a symmetric 95% credible interval of [-0.001 ; 0.025]).

As can be expected, for both T1T3 and T1T4 the PPVser is higher than the PPVpar while the NPVpar is higher then the NPVser.

Table 1: posterior densities for the best fitting model

Parameter	mean	2.5 %	median	97.5 %
Prevalence	0.2994	0.2657	0.2994	0.3339
$sensitivity_{T1}$	0.9953	0.9828	0.9968	0.9999
$sensitivity_{T2}$	0.9869	0.97	0.9881	0.997
$sensitivity_{T3}$	0.9206	0.8596	0.9216	0.9731
$sensitivity_{T4}$	0.9203	0.8594	0.9213	0.973

$specificity_{T1}$	0.9722	0.9449	0.973	0.9938
$specificity_{T2}$	0.9438	0.9148	0.9446	0.9684
$specificity_{T3}$	0.9932	0.9855	0.9937	0.9981
$specificity_{T4}$	0.9984	0.9941	0.9989	0.9999
PPV1	0.9382	0.8774	0.9402	0.9865
PPV2	0.8821	0.82	0.8838	0.9346
PPV3	0.983	0.9639	0.9843	0.9952
PPV4	0.996	0.9852	0.9972	0.9999
NPV1	0.998	0.9925	0.9986	0.9999
NPV2	0.9941	0.9864	0.9947	0.9986
NPV3	0.9666	0.9389	0.9673	0.9894
NPV4	0.9667	0.9391	0.9674	0.9893
sep13	0.9996	0.9985	0.9998	1.0
sep14	0.9996	0.9984	0.9998	1.0
ses13	0.9163	0.8544	0.9174	0.9696
ses14	0.916	0.8547	0.9169	0.9695
spp13	0.9656	0.9376	0.9663	0.9884
spp14	0.9706	0.9434	0.9714	0.9926
sps13	0.9998	0.9995	0.9998	1.0
sps14	1.0	0.9998	1.0	1.0

Pr(D+ | T1+,T3+)	0.9995	0.9986	0.9996	0.9999
Pr(D+ | T1+,T3-)	0.5511	0.1875	0.5569	0.8976
Pr(D+ | T1-,T3+)	0.2152	0.006818	0.1717	0.6428
Pr(D+ | T1-,T3-)	1.664E-4	3.474E-6	1.008E-4	7.114E-4
$PPVpar_{T1T3}$	0.9251	0.8634	0.927	0.975
$NPVser_{T1T3}$	0.9651	0.9372	0.9659	0.988

Pr(D+ | T1+,T4+)	0.9999	0.9995	0.9999	1.0
Pr(D+ | T1+,T4-)	0.5509	0.1879	0.5545	0.8978
Pr(D+ | T1-,T4+)	0.5268	0.03131	0.54	0.9733
Pr(D+ | T1-,T4-)	1.675E-4	3.471E-6	1.01E-4	7.143E-4
$PPVpar_{T1T4}$	0.9353	0.8745	0.9373	0.984
$NPVser_{T1T4}$	0.965	0.937	0.9657	0.9881

DISCUSSION

It is concluded that the both T1T3 and T1T4 combination in a parallel interpretation is the best option for the aim of detecting seronegative animals with more or less equal and very high NPVpar. The T1T4 combination however had a slightly higher PPVpar giving a better all-round post test knowledge (PPVpar+NPVpar). Although this is not directly visible in the summary statistics of both parameters as both credible intervals substantially overlap there is a big confidence of having a higher $PPVpar_{T1T4}$ then a $PPVpar_{T1T3}$. This may seem odd because the mean difference is only 0.01 but is due to the fact that the distributions are heavily skewed (not shown). We should keep in mind however that, if the aim is to maximize the PPV, one would not choose for a parallel interpretation scheme but use a serial instead.

Although one could say that it is not necessary to use any interpretation scheme because we know the PPV and NPV of every test combination it is a fact that in real life the test will be used in sequence and testing will be stopped if the first test is negative (believe the negative; serial) or vice versa when it is positive (believe the positive, parallel) for financial reasons.

Comparison of diagnostic accuracy between different tests based on one cut-off value does not fully represent the diagnostic power of a test (Greiner *et al.*, 2000) and may even be misleading. As an alternative ROC analysis is suggested. In this setup however cut-off values are fixed in the standard operating protocol and cannot be changed. In addition we do not recommended to use this "no gold standard" latent class approach to construct ROC curves.

For this study the latent variable D will rather define a sort of seropositive status of the animal instead of the disease status therefore influencing the conditional probabilities se and sp and the post test probabilities. This may be a major comment as we don't really assess here the diagnostic capacities of the test. We are however convinced that, as the regulations are based on the seronegative status of the animal that the estimates from this model are valid in this framework.

Trying to estimate the "real" diagnostic capacities of the test would require the modelling of higher level dependencies. However, modelling these higher interactions lead inevitably to overparametrisation of the model making it highly prior driven. Next to this, even in a setup with an infinite number of diagnostic tests it is not possible to model all possible dependencies as the lack of df increases exponentially with the addition of tests. Modelling conditional dependencies and/or checking for it by, for example, looking at the conditional log odds ratio (not shown) will therefore always be restricted. This leads to the conclusion that, as stated by Pepe (2003), that the definition of the latent variable D and as a consequence the conditional probabilities based on that parameter will always be dependent on the tests included in the setup which makes it rather subjective. On the other hand, the assumption of a gold standard may even be worse as there is no real definition for D, which makes it a rather artificial way to solve the problem. Keeping this in mind it is reasonable that one should distinguish between D which is unobservable and the diagnosis which is observable (Kraemer (1992).

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MODIFIED EXACT SAMPLE SIZE FOR DIAGNOSTIC TEST PARAMETER ESTIMATION

Geoffrey T. Fosgate[1]

SUMMARY

Accurate sample size estimates based on realistic assumptions are necessary for the design of epidemiologic studies concerning the validation of diagnostic tests. Diagnostic sensitivity and specificity should be estimated, as other population proportions, within a specified level of precision and with the desired level of confidence. Confidence intervals based on the normal approximation to the binomial do not achieve the specified coverage when the proportion is close to the boundary values of 0 and 1. This is a problem for the evaluation of diagnostic tests that have high levels of accuracy. The mid-P modified exact method of confidence interval estimation has better statistical properties for proportions close to the boundaries and therefore a sample size algorithm based on this method was developed to address the limitations of the normal approximation. This algorithm resulted in sample sizes that achieved the appropriate confidence interval width even in situations when normal approximation methods performed poorly.

INTRODUCTION

The calculation of the sample size is an important component of the epidemiologic study design process in general (Kelsey, Whittemore, Evans & Thompson 1996) and specifically for the validation of diagnostic tests (Greiner & Gardner 2000). The ability of the estimated sample size to yield statistically significant findings upon completion of data collection depends upon the choice of the assumptions and the statistical model used to make the calculations. The sample size method chosen should parallel the planned data analysis to the extent possible. The use of overly conservative methods of sample size estimation would result in statistically significant findings; however, such methods would not be efficient due to the increased cost associated with the excessive number of study subjects.

Statistical procedures based on large sample theory (i.e. normal approximation) are not always appropriate when the underlying distribution grossly deviates from normal. Figure 1 depicts the sampling distributions for a proportion of 0.99 with a sample size of 200 based on normal and binomial models. The normal curve extends beyond 1.0, which is not possible for a proportion. Confidence intervals based on the normal approximation methods do not achieve nominal coverage for proportions that are close to the boundary values. Figure 2 shows the coverage probabilities for the normal approximation and the mid-P exact methods of confidence interval estimation for a sample size of 200 and proportions ranging from 0.90 to 0.99.

Newly developed assays, including polymerase chain reaction (PCR) based diagnostics, are often expected to be highly accurate as measured by diagnostic sensitivity (probability of a positive test in a truly affected individual) and specificity (probability of a negative test in a truly non-affected individual). The specificities of such assays are often reported to be greater than or equal to 0.98 (Checkley, Waldner, Appleyard, et al. 2003; Kurabachew, Enger, Sandaa, et al. 2004; Kelley, Zagmutt-Vergara, Leutenegger, Myklebust, et al. 2004; normal approximation to the binomial perform poorly for proportions close to 0 and 1

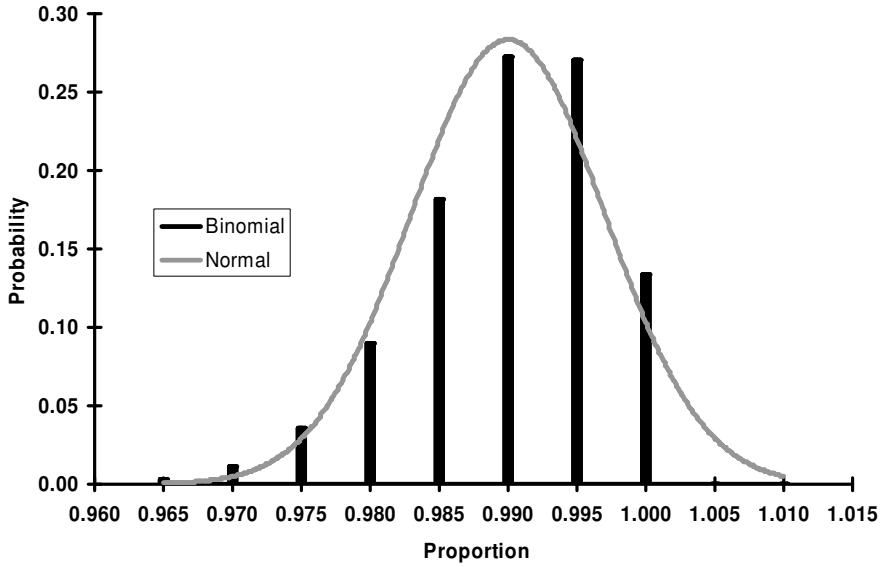

Figure 1. Sampling distributions for the means of a binomial and normal model assuming a true proportion of 0.99 and a sample size of 200.

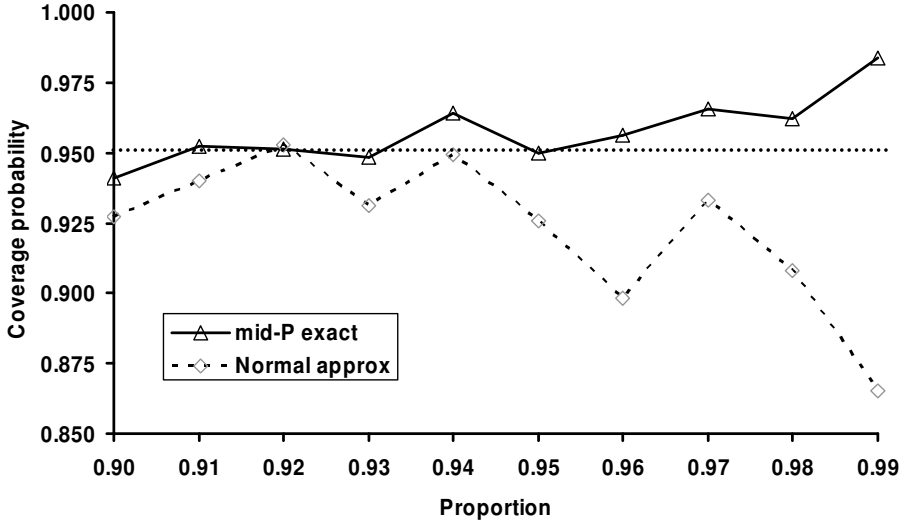

Figure 2. Comparison of coverage probability for 2 methods of 95% interval estimation at multiple proportions relatively close to 1.

(Brown, Cai & DasGupta 2001) and therefore other methods would be recommended for the analysis of data in such instances. Sample size calculations for the evaluation and comparison of diagnostic assays has been the focus of previous reports (White & James 1996; Obuchowski 1998; Alonzo, Pepe & Moskowitz 2002; Knottnerus & Muris 2003), but the design of such studies based on sample sizes calculated using normal approximation methods are not likely to yield results at the desired level of precision. The design of evaluation studies for assays that are expected to be close to perfect would benefit from sample size estimates based on modified exact binomial theory. The objective of the paper reported here was to compare the performance of a modified exact binomial sample size computer algorithm to the usual normal approximation method based on the Wald statistic.

MATERIALS AND METHODS

The sample size algorithm was designed to follow the mid-P method (Lancaster 1961; Berry & Armitage 1995) of adjusting the traditional exact binomial confidence interval. This method is less conservative than true exact methods, yet still achieves good coverage (Brown, Cai & DasGupta 2001; Agresti 2001; Agresti A. 2003; Berry & Armitage 1995; Vollset 1993).

Calculation of the sample size based on this modification is summarized in the following two formulae.

Lower limit

$$\frac{1}{2}\binom{n}{x}p_L^x(1-p_L)^{n-x}+\sum_{k=x+1}^{n}\binom{n}{k}p_L^k(1-p_L)^{n-k}=\alpha/2$$

Upper limit

$$\frac{1}{2}\binom{n}{x}p_U^x(1-p_U)^{n-x}+\sum_{k=0}^{x-1}\binom{n}{k}p_U^k(1-p_U)^{n-k}=\alpha/2$$

Two different sample sizes, one for each of the desired bounds of the confidence interval, will be calculated by solving the formulae above. This problem can be overcome by combining the two probabilities into the single formula below.

$$\binom{n}{x}p_U^x(1-p_U)^{n-x}+\sum_{k=x+1}^{n}\binom{n}{k}p_L^k(1-p_L)^{n-k}+\sum_{k=0}^{x-1}\binom{n}{k}p_U^k(1-p_U)^{n-k}=\alpha$$

Using the above formula for the sample size situation necessitates that p_U and p_L be fixed by the investigator as the desired upper and lower limits of the confidence interval, respectively, around the hypothesized proportion (p_0). The equation is then solved for the value of *n* yielding the appropriate sample size. The value of *x* is calculated as the integer that when divided by *n* yields the closest value to the proportion hypothesized by the investigator ($p_0 \approx x / n$). Solving this single formula often results in unequal probability within each tail of the distribution because the sum of the 2 tails is required to equal alpha, but each tail is not forced to equal alpha/2.

An iterative routine was written in FORTRAN (Compaq Computer Corporation 2002) to implement the sample size equation discussed above. The input for the algorithm is the hypothesized proportion (p_0), the desired error limit (forming the limits p_L and p_U), and the desired level of confidence ($1 - \alpha$). The sample size algorithm starts the procedure at the minimum sample size (*n*) necessary to observe the entered proportion exactly. For example, a proportion of 0.25 would start at $n = 4$ and for a proportion of 0.95 the starting point would be 20. The value of *x* yielding the entered proportion is always 1 at the first iteration of the sample size procedure because of this starting point. The algorithm simply adds 1 to the sample size at each iteration and sums the probabilities in each tail for these values of *x* and *n* (such that $p_0 \approx x / n$). The appropriate sample size has been reached when the sum of the tail probabilities is less than the specified alpha level (1 – confidence).

RESULTS

The sample sizes estimated using the modified exact algorithm were very similar to the normal approximation method for evaluated proportions between 0.5 and 0.8 (data not shown). The width of the mid-P confidence interval formed using the calculated sample sizes from both methods were less than or equal to the nominal width for evaluated proportions between 0.5 and 0.8 (data not shown). The sample sizes calculated for proportions greater than 0.8 were larger using the modified exact method (Table 1) and resulted in confidence interval widths being noticeably different for the 2 methods (Figure 3). The sample sizes

Table 1. Comparison of sample sizes for the estimation of binomial proportions +/- 0.05 (precision) at three levels of confidence using modified exact and normal approximations.

		90% confidence level		95% confidence level		99% confidence level	
Proportion	Limit	Exact	Approx	Exact	Approx	Exact	Approx
0.80	0.05	175	174	249	246	430	425
0.81	0.05	168	167	241	237	420	409
0.82	0.05	161	160	232	227	400	392
0.83	0.05	157	153	222	217	387	375
0.84	0.05	149	146	212	207	368	357
0.85	0.05	140	138	200	196	353	339
0.86	0.05	134	131	192	185	335	320
0.87	0.05	123	123	183	174	315	301
0.88	0.05	116	115	173	163	300	281
0.89	0.05	109	106	161	151	281	260
0.90	0.05	100	98	148	139	260	239
0.91	0.05	97	89	133	126	244	218
0.92	0.05	86	80	124	114	225	196
0.93	0.05	82	71	113	101	213	173
0.94	0.05	66	62	100	87	197	150
0.95	0.05	60	52	97	73	178	127

Exact = sample size calculated using the modified exact computer algorithm
Approx = sample size calculated using the usual large sample normal approximation

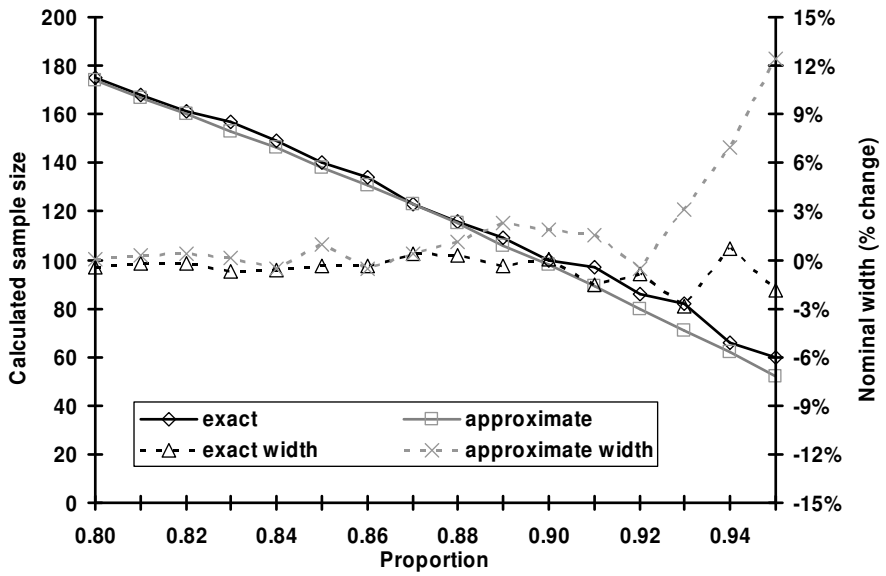

Figure 3. Comparison of sample size estimates for modified exact and normal approximation methods and their ability to achieve nominal width of mid-P confidence intervals. Sample sizes estimated for specified proportion with 0.05 precision and 90% confidence level.

based on the normal approximation resulted in confidence intervals that were often too wide (larger than nominal width). The corresponding intervals for the exact method tended to be narrower than the specified length. The apparent fluctuations in the coverage of the confidence intervals are likely the result of the discrete data and the point estimate (x / n) not being exactly the same for the different sample sizes. The difference in confidence interval width between the modified exact and normal approximation methods becomes more dramatic as the proportion approaches 1 (Figure 4).

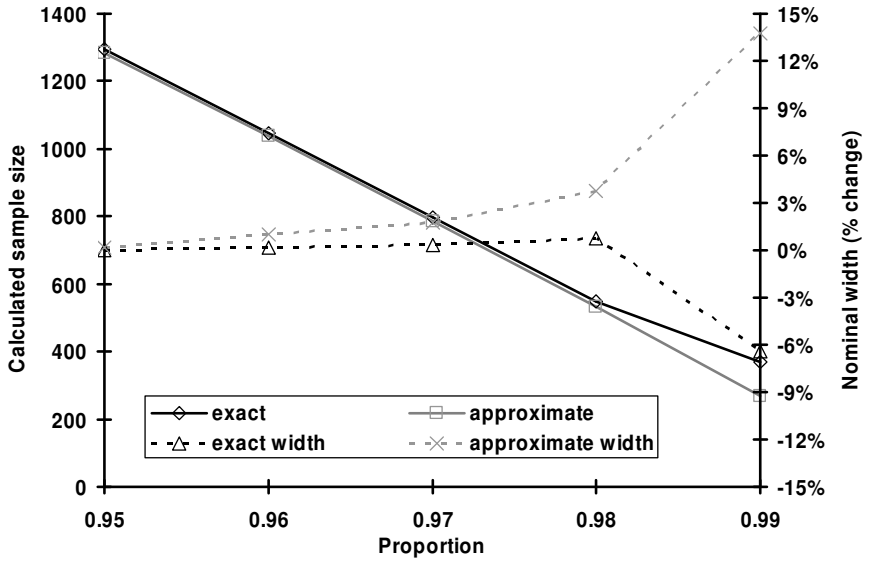

Figure 4. Comparison of sample size estimates for modified exact and normal approximation methods and their ability to achieve nominal width of mid-P confidence intervals. Sample sizes estimated for specified proportion with 0.01 precision and 90% confidence level.

DISCUSSION

The method of sample size estimation is important for the proper design when planning an epidemiologic study concerning the validation of diagnostic tests or the estimation of other population proportions expected to be close to boundary values. The author is not aware of another available sample size procedure for the estimation of a binomial proportion that is not based on usual large sample normal approximation methods. The noted deficiencies in intervals estimated using such methods should caution investigators not to rely on similar formulae when determining the necessary size of a study. The sample size method presented here results in intervals that achieve nominal width (or narrower) even in situations when the hypothesized proportion is relatively close to 1.

The developed computer algorithm is based on the mid-P adjusted exact method and can therefore be computationally intense for certain combinations of proportion, interval width, and confidence level. The algorithm was designed to be most efficient for proportions greater than 0.5 due to the way the computer code was written. The program converts proportions <0.5 to values >0.5 (1- entered proportion) before starting the algorithm for this reason. Proportions less than 0.5 were not evaluated in this paper because sample sizes will be symmetrical. Diagnostic test parameters (i.e. sensitivity and specificity) are also more likely to be close to 1 rather than 0.

The computational intensity of the binomial probability function prevents the sample size algorithm from being able to solve all possible combinations of proportion, interval width, and confidence level. The program will fail to find the correct sample size when individual binomial probabilities approach 1×10^{-310}. When individual probabilities become functionally zero the overall sum of probabilities will start to decrease and the algorithm will continue to cycle without ever finding a suitable sample size. The program was therefore designed to end upon reaching an individual probability of zero. This limitation could be improved by incorporation of variables with higher precision (e.g. 16 byte variables), but these were not available in the computing environment used by the author. This limitation is most severe for proportions close to 0.5 and is not much of an issue as the hypothesized proportion approaches 1. Therefore, the algorithm still appears to function well for most situations in which the normal approximation sample size methods perform poorly ($p > 0.85$).

Finally, the design of diagnostic test evaluation studies where the sensitivity or specificity is expected to be close to perfect would benefit from new sample size methods that are not based on the usual normal approximation. The modified exact method for sample size estimation is an improvement that would facilitate the evaluation of diagnostic tests. It is important to provide newly developed tools to the practicing epidemiologist to allow for there mainstream use.

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ESTIMATING THE PROBABILITY OF FREEDOM OF CLASSICAL SWINE FEVER VIRUS OF THE EAST-BELGIUM WILD-BOAR POPULATION

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INTRODUCTION

Classical swine fever (CSF) is a highly contagious viral infection in pigs and wild boars and is classified as a List A disease by the Office International des Epizooties (OIE). The disease usually causes high morbidity and mortality in domestic-pig populations. In the mid-1980s, the European Union implemented an eradication programme combining a non-vaccination policy with a strategy of stamping out infected herds. These control measures stopped CSF being endemic in most of the EU territory – but epidemics of CSF (with severe socio-economic consequences) have occurred in different European Member States ever since (Elbers et al., 1999; Fritzemeier et al., 2000; Koenen et al., 1996; Mintiens et al., 2003; Miry et al., 1991).

Complete and long-term eradication of CSF in the EU is hampered by the incidence of the infection in wild-boar populations in different Member States. Wild boars are as susceptible to CSF infections as domestic pigs (Depner et al., 1995). As a consequence, wild boars represent an important risk (indifferent to geographical borders) for infecting the domestic-pig population. From different studies conducted from 1993 to 1997, Fritzemeier et al. (2000) estimated that 59% of the primary outbreaks of CSF in domestic pigs in Germany were related to direct or indirect contact with infected wild boars. The tradition to keep domestic pigs at free range – and, consequently, in close contact with the wild-boar population – is considered a major cause of CSF-outbreaks in some areas of Sardinia (Laddomada, 2000). Lowings et al. (1994) found evidence for the spread of CSF-virus from initially infected captive to free-living wild boars and further to domestic pigs in Italy from 1985 to 1991.

In 1998, several CSF-outbreaks in domestic pigs and wild boars were identified in the Rheinland-Palatinate region of Germany at a 1- to 50-km distance from the Belgian eastern border. These new cases alerted the Belgian Veterinary Services, which implemented a surveillance programme in the same year. The objective of the programme was to monitor whether the Belgian wild-boar population remained free of CSF and to take effective measures to protect the domestic pig population in case CSF-infections in wild boar would occur. The treat of CSF-introduction into the Belgian wild-boar population has not diminished in recent years and the surveillance programme continues.

A report of the Scientific Committee on Animal Health and Animal Welfare of the European Commission (CEC, 1999) includes recommendations for setting up monitoring programmes for CSF-infection in a wild-boar population. These recommendations are based on a method described by Cannon and Roe (1982), which allows calculating the exact probability of detecting infected populations. The method assumes a minimal expected prevalence of infected animals in an infected population. The aforementioned EC report assumes that one would detect at least a 5% prevalence in a CSF-infected wild-boar population. This assumption, however, is not based on any

experimental or epidemiological evidence. Therefore, the proposed methodology could overestimate the probability of a wild-boar population being free of CSF-virus if the true, non-zero prevalence were < 5%.

We describe the results of the surveillance programme over a period from October 1999 to December 2001. Additionally, we propose an alternative method to provide evidence for the East-Belgian wild-boar population being free of CSF and evaluate the efficiency of the surveillance programme.

MATERIALS AND METHODS

The Surveillance Programme

The surveillance programme started in 1998 and involved the monitoring of wild boars for CSF-infections within the three provinces (Namur, Liège and Luxembourg, total 13,798 km^2), which mutually include 95% of the Belgian wild-boar population (Fig. 1). The programme consisted of the examination on a voluntary basis of a convenience sample of wild boars that were killed or found dead by hunters. Per province, a minimal annual number of 100 animals to be examined was prescribed.

From January 2000 onwards, the programme was extended by dividing the target area into two zones and by implementing additional control measures (Fig. 1).

- A surveillance zone (~ 125 km2) bordering the CSF-affected area in Germany was defined. This zone was assumed to have higher risk for introduction of CSF-virus. Therefore, all wild boars that were killed or found dead were required to be examined for the presence of CSF-infection and movement restrictions for domestic pigs were implemented.
- In the screening zone (the remaining parts of the three provinces and with lower risk for CSF-virus introduction) the monitoring of wild boars killed or found dead continued on a voluntary basis. For only Liège and Luxembourg provinces, an additional 50 animals were to be examined every year in the communities bordering Germany and the Grand Duchy of Luxembourg.

Sample Collection

Blood samples and lymph nodes were collected from each wild boar that was examined within the surveillance programme. If sampling lymph nodes was not possible, kidneys were used. All samples were collected at the Control Centre of Loncin and dispatched to the Veterinary and Agrochemical Research Centre in Brussels for further analysis.

For each animal that was sampled, additional information was recorded (e.g. sampling date, postal code of the municipality in which the animal was shot or found dead, sex, age group (young, sub-adult, adult), estimated weight).

Diagnostic Methods

The collected samples received three types of diagnosis:

Antibody detection: A serial testing procedure was performed to detect antibodies against CSF-virus in serum samples. As a first step in this procedure, a competitive enzyme-linked immunoassay (ELISA) test (HerdChek CSFV antibody ELISA, IDEXX) was used. Samples that tested positive by the ELISA test were then simultaneously examined by a CSF-virus neutralisation test and a BVD-virus neutralisation test, based on the neutralisation assay (NPLA) as described by Holm Jensen (1981). Only samples that tested positive result the ELISA and the CSF-virus neutralisation test and negative for the BVD-virus neutralisation test were reported to contain antibodies against CSF-virus. For all other samples, it was concluded that antibodies against CSF-virus were absent.

Virus detection: Organ-tissue suspensions were inoculated on a monolayer of sub-confluent PK15 cells, cultivated on multi-cup plates to isolate CSF-virus. The virus was identified by an anti-CSF immunoglobulin conjugated at fluorescein isothiocianate (Koenen et al., 1996).

Virus RNA detection: A single-tube RT-nPCR test was performed to detect CSF-virus RNA in tissue samples (McGoldrick et al., 1999).

To our knowledge, no information is available on the sensitivity and specificity of these three diagnostic methods.

Freedom of CSF-virus

Because of a lack of knowledge on the dynamics of CSF-virus in a wild-boar population, a minimal expected prevalence resulting from an introduction of CSF-virus could not be selected and justified. Hence, the commonly used methods described by Cannon and Roe (1982) or by Cameron and Baldock (1998) for calculating the probability of freedom of disease in a population was not applicable. However, estimating the prevalence of CSF-infected wild boars in the Belgian population would provide evidence on the absence of the virus in the population and would give input to a surveillance programme, suitable for the Belgian situation.

Using Bayes theorem, the posterior probability of freedom of CSF-virus (F) given the observed test results (T) can be derived:

$$P(F|T) = \frac{P(T|F)P(F)}{P(T)} \quad (1)$$

When the observed test results are obtained by applying a diagnostic test to a survey sample, one can estimate the probability of freedom of CSF-virus (F), the prevalence given the population is not free ($prev$), and the sensitivity (Se) and specificity (Sp) of the diagnostic test given the data. Equation (1) can than be extended:

$$P(F, prev, Se, Sp|T) = \frac{P(T|F, prev, Se, Sp)P(F, prev, Se, Sp)}{P(T)}$$

Using Bayesian inference, the posterior probability distributions for parameters *F, prev, Se* and *Sp* are estimated by a multinomial likelihood function and by including prior information on the four parameters. The multinomial likelihood for a positive test result can be written as:

$$P(T|F, prev, Se, Sp) = (1-F)(prev \times Se + (1-prev)(1-Sp) + F(1-Sp) \quad (2)$$

The likelihood function based on (2) takes into account that the observed prevalence only occurs when the population is not free of CSF-virus and can under on following assumptions be extended to a test result based on 3 test methods.

1. The results of all diagnostic methods, which were used in this study, are independent conditional on the infection status of the tested animals (all three methods detect different biological phenomena's).
2. The probability of a negative test result given that an animal is non-diseased is the same in a free or non-free population.
3. The probability of having a non-free population given that an animal is infected is equal to 1.
4. The CSF-prevalence in the population equals the prevalence given that the population is not free of CSF-virus times the probability that the population is not-free of CSF-virus.

In our study, the prevalence was calculated based on the samples that were collected from wild boars within the surveillance programme from October 1999 to December 2001 (and analysed by all three diagnostic methods). Any qualitative superiority of one of the methods to the two others was ignored (no-gold standard method). Using the three diagnostic methods in parallel, 8 different combinations of test results could be obtained, when assuming that results of all diagnostic methods are independent conditional on the infection status of the tested animals. For each of 8 test result combinations, the likelihood function to observe a given test result combination was formulated, based on the sensitivity (Se_i) and specificity (Sp_i) of each of the three testing methods, the probability of freedom of CSF-virus (F) and the CSF-prevalence ($prev$) in the population when it would not be free of the virus (Table 1). This resulted in 8 equations with 8 unknown parameters. Posterior densities for the 8 unknown parameters (Se_i and Sp_i for i= 1 to 3; F, and $prev$), were obtained applying Bayesian inference using Gibbs sampling (Gelfand and Smith, 1990) in the

WinBugs software (version 1.4). Three parallel sequences with different starting values were run for each model and convergence was monitored with the Gelman-Rubin test. To assure stable posterior density outcomes, all models ran for another 10,000 iteration after the Gelman-Rubin test converged to 1.

The model had 7 degrees of freedom while a total of 8 parameters had to be estimated. This made it unidentifiable without prior information on at least one parameter. No clear prior information on the parameters could be obtained from literature or reliable sources. Therefore different combinations of prior information for the different parameters were used to analyses the sensitivity of the obtained prior distributions to the choice of their priors.

RESULTS

Descriptive statistics

A total of 1,282 animals was sampled from October 1999 to December 2001 (1,201 from the screening zone and 81 from the surveillance zone). Most were collected during the consecutive annual hunting campaigns (mid-October to end-December). The sampled animals were nearly equally distributed over the age groups and sex categories across the 4 years.

Blood samples of 889 animals were analysed using the described serological methods. Nine samples were positive for antibodies against CSF-virus, 850 samples were negative (for 30 samples, no distinct test result could be derived). Tissue samples of 1,183 animals were examined by the inoculation method and all were CSF-virus negative. Tissue samples from all 1,282 animals were examined by the single tube RT-nPCR test and all were negative. Samples of 789 animals were examined by all 3 diagnostics methods. Nine (of the 789) were sero-positive but all other diagnostic results were negative.

Parameter estimations and sensitivity analysis

The diagnostic results for the 789 animals that were examined in parallel by all 3 diagnostics methods were used for estimating the 8 parameters. Seven different combinations of prior information for the sensitivity and specificity of the diagnostic methods and for the prevalence, given de Belgian wild boar population was CSF-infected, where chosen to run the model. For all these combinations, the posterior median estimates with their 95% credibility intervals for the 8 parameters are summarized in Table 2.

For the prior distribution, the likelihood and the posterior distribution when choosing the least informative prior information (Se & $Sp \geq 0.5$; non-informative prior for *prev*; combination 1), the posterior median probability for freedom of disease was estimated at 0.970 with a 95% credibility interval of 0.149 to 1.000.

DISCUSSION

To obtain unbiased parameter estimates, individuals in a study population should have equal probability to be selected for a survey sample (simple random sample). But, selecting a simple random sample from a wildlife population is almost impossible since it requires the identification of each individual in the population. In our study, parameter estimates are based on a convenience sample of wild boars that were killed or found dead by hunters. This may have led to an overestimation of the prevalence and an underestimation of the probability for freedom of disease, since CSF-infected animals may be ill and weak, which may give them a higher probability to be found dead or shot by hunters. However, the obtained estimate for the probability for freedom of CSF-virus is extremely high. Therefore the number of CSF-infected animals is expected to be very low and a possible selection bias is neglectable.

The sampling probability was also higher for animals in areas with higher risk for introduction of CSF-virus (surveillance zone and the communities bordering Germany and the Grand Duchy of Luxembourg). Moreover, the samples, which had a positive serological result, were obtained from

animals from these high-risk areas. This means that the probability for freedom of CSF-virus in the population of the whole study area (surveillance zone and screening zone) is expected to be higher than the obtained estimate. Because of a lack of detailed information on the size of the wild-boar population in the different areas, separate estimations per area were not possible.

The method that was used to estimate probability of freedom of CSF-virus did not assume that the information provided by one diagnostic method was superior to the others (absence of gold standard) and the probability was calculated considering the quality of the three diagnostic methods for the situation of very low disease prevalence. However, this situation required prior knowledge to be included for the model to convert. The posterior distributions of most parameter were insensitive to the choice of prior information for the sensitivity and specificity of the diagnostics methods. Only the posterior distributions of the sensitivity of the diagnostic methods depended highly on its prior distributions. This was expected since almost all test results in the survey sample were negative. It shows that it is impossible to obtain a reliable estimate of the diagnostic sensitivity in a population with low or absent disease prevalence. The choice of the prior distribution for the prevalence, given the population is free of CSF-virus had an influence on the posterior distribution of the probability of freedom of disease. Choosing a more informative prior narrowed the 95% credibility interval of the posterior distribution and pushed the median towards 1. This can be explained by the fact that we assumed that the CSF-prevalence in the population equals the prevalence given that the population is not free of CSF-virus (*prev*) times the probability that the population is not-free of CSF-virus (*F*). Under this assumption, any increase of *prev* would result in an increase of *F* since the CSF-prevalence in the population is constant.

The aim of the surveillance programme is to provide evidence for the East-Belgian wild-boar population to be free of CSF-virus. As an alternative to the methodology proposed in the report of the European Commission concerning CSF in wild boar (CEC, 1999), we calculated the probability of freedom of CSF-virus, without assuming a minimal expected prevalence when CSF would be present in the population. Independent on the choice of the prior information, all posterior distributions for the probability of freedom of CSF-virus are lying close to the upper boundary of 1. This represents a big gain of knowledge since we did not use any prior information for the probability of freedom of CSF-virus and took the uncertainty about the accuracy of the diagnostic methods into account.

CONCLUSIONS

The results obtained from October 1999 to December 2001 within the frame of the surveillance programme for CSF-virus in the Belgian wild-boar population were sufficient to provide a high probability ($p > 0.97$) that the population in the Namur, Liège and Luxembourg provinces is free of CSF-virus.

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Table 1. Likelihood contributions to observe a given combination of test results when using 3 diagnostic methods in parallel

Test-result combination			Likelihood contribution		
T_1	**T_2**	**T_3**			
+	**+**	**+**	$(1-F)*(prev*Se_1*Se_2*Se_3+(1-prev)*(1-Sp_1)*(1-Sp_2)*(1-Sp_3))$	+	$F*(1-Sp_1)*(1-Sp_2)*(1-Sp_3)$
+	**+**	**–**	$(1-F)*(prev*Se_1*Se_2*(1-Se_3)+(1-prev)*(1-Sp_1)*(1-Sp_2)*Sp_3)$	+	$F*(1-prev)*(1-Sp_1)*(1-Sp_2)*Sp_3$
+	**–**	**+**	$(1-F)*(prev*Se_1*(1-Se_2)*Se_3+(1-prev)*(1-Sp_1)*Sp_2*(1-Sp_3))$	+	$F*(1-prev)*(1-Sp_1)*Sp_2*(1-Sp_3)$
+	**–**	**–**	$(1-F)*(prev*Se_1*(1-Se_2)*(1-Se_3)+(1-prev)*(1-Sp_1)*Sp_2*Sp_3)$	+	$F*(1-prev)*(1-Sp_1)*Sp_2*Sp_3$
–	**+**	**+**	$(1-F)*(prev*(1-Se_1)*Se_2*Se_3+(1-prev)*Sp_1*(1-Sp_2)*(1-Sp_3))$	+	$F*(1-prev)*Sp_1*(1-Sp_2)*(1-Sp_3)$
–	**+**	**–**	$(1-F)*(prev*(1-Se_1)*Se_2*(1-Se_3)+(1-prev)*Sp_1*(1-Sp_2)*Sp_3)$	+	$F*(1-prev)*Sp_1*(1-Sp_2)*Sp_3$
–	**–**	**+**	$(1-F)*(prev*(1-Se_1)*(1-Se_2)*Se_3+(1-prev)*Sp_1*Sp_2*(1-Sp_3))$	+	$F*(1-prev)*Sp_1*Sp_2*(1-Sp_3)$
–	**–**	**–**	$(1-F)*(prev*(1-Se_1)*(1-Se_2)*(1-Se_3)+(1-prev)*Sp_1*Sp_2*Sp_3)$	+	$F*(1-prev)*Sp_1*Sp_2*Sp_3$

with :

+ , –	Test result for the i-th diagnostic method
F	Probability for freedom of CSF-virus
prev	Prevalence of the infection in the study population, given that the population is not free of CSF-virus
Se_i	Diagnostic sensitivity of the i-th diagnostic method
Sp_i	Diagnostic specificity of the i-th diagnostic method

Table 2. The median probability for freedom of CSF-virus (F), the median prevalence of the infection in the study population, given that the population is not free of CSF-virus (prev) and the median diagnostic sensitivity (Se) and specificity (Sp) for the different diagnostic methods, with 95% credibility intervals, using different combination (Comb) of prior information for Se, Sp and prev.

Comb	F	Prev	Antibody detection		Virus detection		Virus RNA detection	
			Se	Sp	Se	Sp	Se	Sp
1	0.970 (0.149–1.000)	0.026 ($3x10^{-4}$–0.824)	0.750 (0.513–0.988)	0.988 (0.979–0.994)	0.745 (0.513–0.987)	0.999 (0.995–1.000)	0.743 (0.512–0.987)	0.999 (0.995–1.000)
2	0.973 (0.155–1.000)	0.026 ($2x10^{-4}$–0.837)	0.945 (0.881–0.981)	0.984 (0.975–0.991)	0.945 (0.883–0.980)	0.995 (0.988–0.998)	0.945 (0.882–0.980)	0.995 (0.988–0.998)
3	0.973 (0.184–1.000)	0.027 ($3x10^{-4}$–0.818)	0.991 (0.955–1.000)	0.989 (0.980–0.995)	0.991 (0.955–1.000)	0.999 (0.996–1.000)	0.991 (0.956–1.000)	0.999 (0.996–1.000)
4	0.996 (0.957–0.999)	0.227 (0.054–0.929)	0.750 (0.512–0.987)	0.988 (0.978–0.994)	0.738 (0.511–0.987)	0.999 (0.995–1.000)	0.740 (0.511–0.988)	0.999 (0.995–1.000)
5	0.988 (0.594–1.000)	0.077 (0.003–0.385)	0.751 (0.513–0.988)	0.988 (0.979–0.994)	0.742 (0.512–0.987)	0.999 (0.995–1.000)	0.742 (0.512–0.987)	0.999 (0.995–1.000)
6	0.989 (0.625–1.000)	0.079 (0.003–0.382)	0.945 (0.884–0.981)	0.984 (0.975–0.991)	0.945 (0.883–0.980)	0.995 (0.988–0.998)	0.945 (0.883–0.988)	0.995 (0.988–0.998)
7	0.989 (0.620–1.000)	0.078 (0.003–0.384)	0.992 (0.955–1.000)	0.989 (0.981–0.995)	0.992 (0.955–1.000)	0.999 (0.996–1.000)	0.991 (0.955–1.000)	0.999 (0.996–1.000)

Combination:

1 Se & Sp >= 0.5 and non-informative prior for prev

2 Se & Sp = beta(80, 5) and non-informative prior for prev

3 Se & Sp = beta(80, 1) and non-informative prior for prev

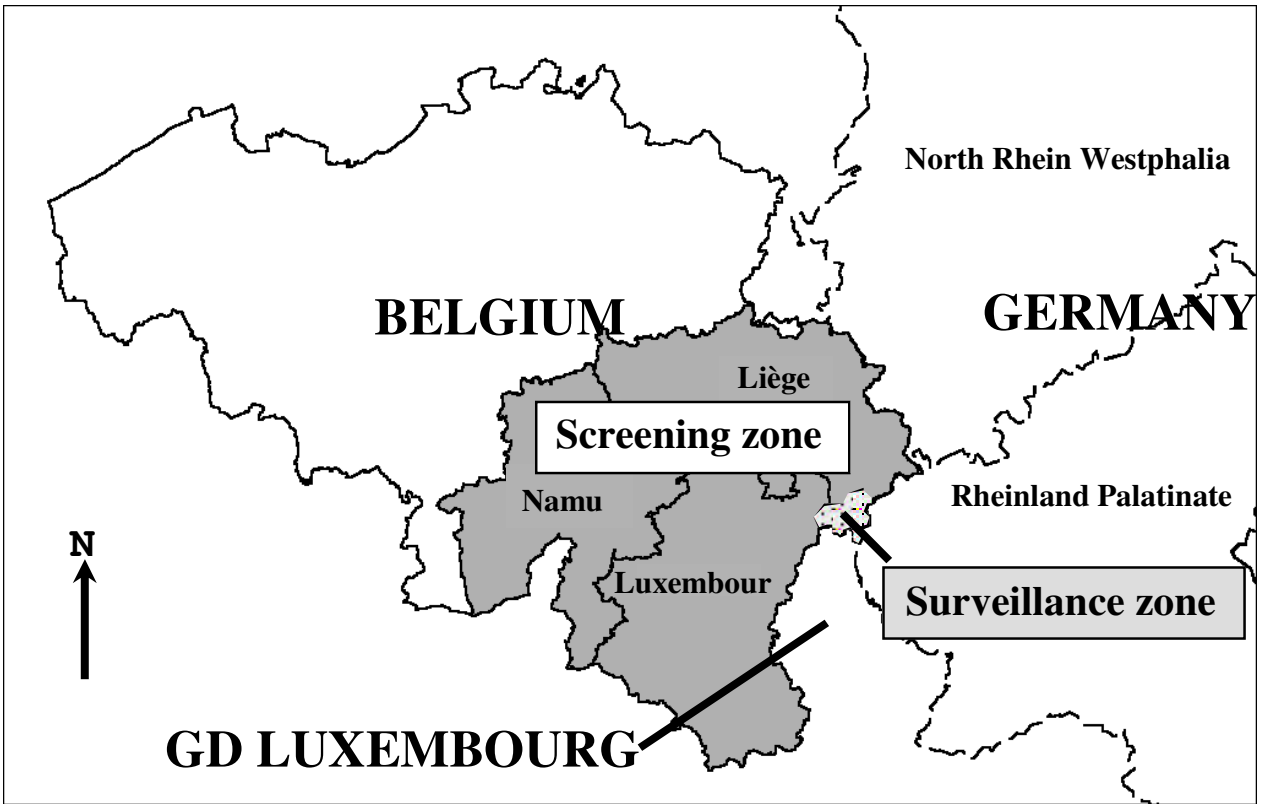
4 Se & Sp >= 0.5 and prev >= 0.05

5 Se & Sp >= 0.5 and prev = beta(1.85, 7)

6 Se & Sp = beta(80, 5) and prev = beta(1.85, 7)

7 Se & Sp = beta(80, 1) and prev = beta(1.85, 7)

Fig. 1. Map of Belgium indicating the screening and surveillance zone for CSF in wild boar.



DURATION OF ANTIBODIES TO THE NON-STRUCTURAL PROTEINS AFTER INFECTION WITH FOOT AND MOUTH DISEASE VIRUS AND COMPARISON BETWEEN VARIOUS SEROLOGICAL TESTS

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ABSTRACT

Two groups of two cattle each were infected with a SAT1 and SAT2 strain respectively and serologically tested for the presence of antibodies to the structural and non-structural proteins (NSPs) over time. It was found that antibodies to the NSPs formed later in the course of infection than antibodies to the structural proteins, and the former were of short duration. Antibodies to the NSPs could only be detected between 10 and 147 days post infection for the two viruses used in the study. Probang samples taken from cattle at intervals demonstrated that virus was present in the oro-pharyngeal regions only up to 14 days indicating that none of the animals had become carriers. Several commercial kits available for the detection of antibodies to the NSPs were compared for sensitivity and specificity using sera from this experiment and poor correlation was found between results.

INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious viral infection of cloven-hoofed animals causing severe, clinically acute vesicular disease. Foot-and-mouth disease virus (FMDV) is a member of the genus *Aphthovirus* and the family *Picornaviridae* (King, 2000) and consists of a small non-enveloped virus with an 8.5-kbp genome which codes for structural as well as nonstructural proteins (NSPs) (Carrol *et al*., 1984; Forss *et al*., 1984). There are seven serotypes recognized worldwide with SAT1, SAT2 and SAT3 predominant in the southern African region (Vosloo *et al*., 2002). Serotypes are clinically indistinguishable with considerable variation in the disease presentation depending on the strain within a serotype, the species affected and previous exposure (Kitching 2002, Kitching & Hughes, 2000; Kitching & Alexandersen, 2000). It is one of the most important economic diseases of livestock owing to both the production losses caused by clinical disease and the disruption caused in international trade with disease-free countries.

In countries or zones seeking freedom from FMD where vaccination is used to control the disease, it is essential to distinguish between infected and vaccinated animals (DIVA). Generally, antibodies to capsid proteins are produced in both infected and vaccinated animals, and antibodies to NSPs are produced only in infected animals. Antibodies against virus-infection associated antigen, for example, have been used since 1966 as serological markers to distinguish between convalescent and vaccinated animals (Lee & Jong, 2004). However, owing to the partially purified nature of commercially available vaccines containing traces of this antigen as well as other NSPs such as 3A, 3B and 3C, healthy vaccinated animals may develop antibodies to these antigens in the absence of infection (Lee & Jong, 2004).

In May 2002 the Office International des Epizooties (OIE) updated its International Animal Health Code in light of advances in diagnostic tests, allowing countries that vaccinate in the face of an outbreak of FMD to regain disease-free status after 6 months if they can differentiate vaccinated

from infected or convalescing animals. Differentiation of convalescing or infected animals is based on identifying antibodies to the NSPs of FMDV (Berger *et al*., 1990; De Diego *et al*., 1997; Sorenson *et al*., 1998b; Sorenson *et al*., 1992). As these tests will be used in the regulation of trade in live animals and their products, their evaluation is essential.

This paper describes the serological results obtained from cattle infected with two of the five virus strains used in this study, SAT1 NIG 5/81 and SAT2 UGA 02/02, under experimental conditions. The duration of antibodies against structural and NSPs was measured and compared using different commercial and non-commercial kits.

MATERIALS AND METHODS

Experimental animals and sampling procedures. Conventionally reared sexually mature Dexter bulls of 9-24 months and Landrace X pigs were used. The animals arrived at the Exotic Diseases Division – Onderstepoort Veterinary Institute five to seven days before the experiment initiated. Each group consisted of two tethered cattle and one pig (that was allowed to roam freely) and was housed in separate stables. The cattle were examined and body temperature and clinical signs were recorded daily. Heparinised blood, serum and oesophageal-pharyngeal (O/P) scrapings were collected before the experiment started. During the experiment, heparinised blood was collected daily for the first 14 days, serum daily for the first 14 days and weekly after that. O/P scrapings were collected every 14 days from onset of pyrexia using the probang cup (van Bekkum *et al*., 1959; Sutmoller & Gaggero, 1965; Burrows, 1966), vesicular fluid and epithelium were taken from lesions when they appeared. The day of temperature increase in cattle was recorded as Day 0 - first day of clinical signs.

Viruses used in the study and method of infection. FMDV SAT1 NIG 5/81 and SAT2 UGA 02/02 were used to challenge each group respectively. The challenge viruses were titrated on IBRS2 cells and an infection chain was started in each group by inoculation of the pig with 10^5 $TCID_{50}$ FMDV in the bulb of one heel as described previously (De Leeuw *et al*., 1979). The cattle were thus infected by aerosol generated by the infected pigs. Pigs were euthanised as soon as infection was observed in cattle and the latter were euthanised when antibodies to the NSPs had declined to negative levels using the Checkit ELISA kit: Group 1 – (SAT1 NIG 5/81) after 147 days and Group 2 – (SAT2 UGA 02/02) after 98 days.

Virus isolation. Primary pig kidney cell cultures were inoculated with 10% suspensions (w/v) of epithelium and observed for cytopathic effect (CPE) after 48 hours. Cultures that were negative for CPE were passaged blind and investigated for CPE for another 48 hours before results were recorded.

Serological assays used to detect antibodies to the structural and NSPs. The liquid-phase blocking ELISA (lpbELISA) was carried out as described by Hamblin *et al*. (1986) to detect antibodies to the structural proteins. Titres were calculated as that dilution of the serum that inhibits 50% of the virus reaction. All sera were examined for the presence of antibodies to the NSPs using four commercially available ELISA kits: CHEKIT FMD-3ABC (Bommeli Diagnostics, Switzerland), Ceditest® FMDV-NS (Cedi-diagnostics BV, Lelystad, Netherlands), SVANOVIR™ FMD 3ABC-Ab (Svanova Biotech AB, Uppsala, Sweden) and UBI FMDV NS EIA (United Biomedical Inc., New York, USA). Each kit was used according to the manufacturers' instructions.

Detection of viral genomic material in clinical material. Viral RNA extraction, cDNA synthesis and PCR were performed directly on clinical material using methods described previously by Bastos (1998).

Statistical analysis. The measurement of agreement between tests for NSPs was based on kappa statistics. Agreement was classified by values of kappa statistics into almost perfect (0.81 or higher), substantial (0.61–0.80), moderate (0.41–0.60), fair (0.21–0.40), slight (0–0.20) and poor (0) (Thrusfield, 1995).

RESULTS

Clinical signs. The two needle-infected pigs demonstrated temperature increases 1 or 2 days post challenge followed by development of FMD lesions one day later. The cattle infected by aerosol route developed clinical signs 2 to 3 days after they demonstrated temperature increases with Group 1 displaying mouth lesions only while Group 2 had both feet and mouth lesions.

Duration of infection as determined by virus isolation and detection of genomic material. FMDV was isolated from heparinised blood between 3 to 6 days after the onset of clinical signs and at day 14 from O/P scrapings in Group 1, while in Group 2 virus was isolated from heparinised blood at day 1 and 2 with no virus at day 14 in the O/P scrapings. Subsequent O/P samples taken from both groups were negative for virus indicating that the animals did not become carriers of the virus. Similar results were obtained with genomic amplification.

Serological responses to the structural and NSPs. All animals were sero-negative at the onset of the experiment (results not shown). Significant titers of circulating antibodies to the structural proteins were detected at day 9 for Group 1 and days 2-5 for Group 2 after the first appearance of clinical signs (Table 1). SAT1 NIG 5/81 infected animals remained sero-positive beyond 147 days and SAT2 UGA 02/02 infected animals beyond 98 days.

The sensitivity of four commercially available kits that test for the presence of antibodies to the NSPs was compared. Results obtained with the Chekit and Cedi kits are indicated in Table 1. The SVANOVIR kit showed an intermittent antibody response while the UBI kit did not detect sero-conversion (data not shown). The SAT1 NIG 5/81 infected animals were sero-positive between days 21 and 84 and one single reaction was observed at day 133 using the Checkit kit, while sero-conversion was detected from day 11 until 147 using the Cedi kit (Table 1). Animals infected with SAT2 UGA 02/02 were sero-positive between days 12 and 42 using the Checkit kit compared to days 10 to 21 with the Cedi kit.

Table 1. Detection of antibodies to FMDV infection using the lpbELISA to detect antibodies to the structural proteins and the Chekit and Cedi kits to detect antibodies to the NSPs.

Day	***Week***	***LPB ELISA***				***Checkit***				Cedi			
		SAT1 Nig 5/81		***SAT2 Uga 02/02***		***SAT1 Nig 5/81***		***SAT2 Uga 02/02***		***SAT1 Nig 5/81***		***SAT2 Uga 02/02***	
		1	***2***	***3***	***4***	***1***	***2***	***3***	***4***	***1***	***2***	***3***	***4***
1		-	-	-	-	-	-	-		-	-		-
2		-	-	+	-	-	-	-		-	-		-
3		-	-	+	-	-	-		-	-	-	-	-
4		-	-	+	-	-	-	-		-	-	-	-
5		-	-	+	+	-	-	-	-	-	-	-	-
6		-	-	+	+	-	-	-	-	-	-	-	-
7	1	-	-	+	+	-	-	-	-	-	-	-	-
8		-	-	+	+	-	-	-	-	-	-	-	-
9		+	+	+	+	-	-	-	-	-	-	-	-
10		+	+	+	+	-	-	-	-	-	-	+	-
11		+	+	+	+	-	-	-		+	-	+	-
12		+	+	+	+	-	-	+	-	+	-	+	-
13		+	+	+	+	-	-	-	+	+	-	+	-
14	2	+	+	+	+	-	-	-	+	+	-	+	-
21	3	+	+	+	+	-	+	-	-	+	+	+	+
28	4	+	+	+	+	-	+	-	+	+	+	-	-
35	5	+	+	+	+	-	+	-	+	-	+	-	-
42	6	+	+	+	+	+	-	-	+	+	+	-	-
49	7	+	+	+	+	-	+	-	-	+	+	-	-
84	8	+	+	+	+	+	-	-	-	+	+	-	-
98	9	+	+	+	+	-	-	-	-	+	+	-	-
105	10	+	+	+	+	-	-	-	-	+	-	-	-
119	11	+	+	+	+	-	-	-	-	+	+	-	-
126	12	+	+	+	+	-	-	-	-	+	+	-	-
133	13	+	+	+	+	-	+		-	+	+	-	-
140	14	+	+			-	-		-	+	+	-	-
147	15	+	+			-	-			+	+		

Sero-negative
\+ Sero-positive

Comparison of the various kits used to detect antibodies to the NSPs. The test agreement based on kappa statistics is indicated in Table 2. The agreement was substantial (0.784) and moderate (0.429) for the Cedi/SVANOVIR kits in the two cattle groups and fair to poor between the other kits.

Table 2. Agreement between tests for the detection of antibodies to the NSPs using kappa statistics.

Tests	**Kappa values**	
	SAT1 NIG 5/81	***SAT2 UGA 02/02***
Bommeli/Cedi	0.080	0.134
Bommeli/SVANOVIR	0.078	0.243
Bommeli/UBI	0.000	0.000
Cedi/SVANOVIR	0.784	0.429
Cedi/ UBI	0.000	0.000
SVANOVIR/UBI	0.000	0.000

DISCUSSION

Previous studies have shown that antibodies to FMDV polyproteins 3ABC and 3AB are the most reliable indicators of previous infection in vaccinated populations (Bergmann *et al*., 1993; De Diego *et al*., 1997; Mackay, 1998; Mackay *et al*., 1998; Sorensen *et al*., 1998a) with the added advantage that a single test can be used to detect antibodies to any of the seven serotypes of FMDV (Bronsvoort *et al.*, 2004). The aim of this study was to compare the ability of four commercially available ELISAs to detect antibodies directed against the NSPs of FMDV SAT1 and SAT2 during the course of natural infection in cattle.

There was a delay in the anti-3ABC kinetics compared with the antibody response against serotype specific structural proteins (Table 1). Rather than representing differences in the sensitivity between tests for structural and NSPs, this difference in the kinetics more likely reflects differences in the immunogenicity of structural and NSPs (Bruderer *et al*., 2004). This implies that the ELISAs used to detect antibodies to the NSPs cannot be utilised int the early phases of an outbreak. Previous studies have suggested that the antibody response to the 3ABC proteins occurs slightly later than the response to the structural proteins (Sorensen *et al*., 1998a, b) and that the 3ABC antibodies may not persist for as long as the structural proteins antibodies. The estimates range from only a few months (8) up to 3.5 years (Chung *et al.*, 2002) for the 3ABC antibodies, compared to 1 to 3 years (Bachrach, 1968) or even up to 4.5 years (Cunliffe, 1962) for the antibodies directed to the structural proteins. In this study, antibodies to the NSPs to one isolate lasted only 42 days, significantly shorter than what was found the classical European serotypes of FMDV.

Based on data in Table 1, the Cedi-test kit had the earliest and longest detection rate of FMDV NSPs but although it detected antibodies earlier in the SAT2 infection (days 10-21) than the Chekit kit, the latter detected antibodies for a longer period (days 12-42). Intermittent patterns of anti-NSP antibody response were observed, but this was more pronounced with the SVANOVIR test kit (not shown). The statistical agreement between the kits was moderate for the Bommeli/Cedi kits, fair to poor for the other combinations. Standardisation of cut-off points and increased sample size can improve performance of these tests (Moonen *et al.*, 2004; Bronsvoort *et al.*, 2004). The current recommendation by the OIE is a combination of a 3ABC ELISA and a subsequent enzyme-linked immunoelectrotransfer blot assay to confirm all positives (Bergmann *et al*., 2000). Such an approach has been used successfully in South America, where vaccination has been used for eradication (Bergmann *et al*., 1996).

The difference in sensitivity between the tests could be ascribed to the antigen used in the test. The UBI kit incorporates peptides of 3B only, while the other kits utilise different combinations of the 3ABC polypeptide obtained from the European serotypes. Sequence analysis of the 3C proteinase genes of various FMDV isolates from six serotypes indicated that the SAT serotypes differed significantly from the European serotypes (van Rensburg *et al*., 2002) and it is possible that the antigen in the kits are antigenically different from the antigens present in the field.

At present there is no fully validated, OIE-approved test capable of making a distinction between antibodies to structural and NSPs. Although probang sampling can identify carrier animals, it cannot be used to exclude the possibility of carriers because (a) the amounts of virus are low (often close to the limits of detection of cell culture assay systems) and decline over time, and (b) the excretion of virus by carriers is intermittent (Alexandersen *et al.*, 2003). The present study also showed that the tests for NSP detected animals that had been infected even though oesophageal-pharyngeal scrapings were negative for FMDV. Moreover, no statistical sample frame has been established for probang sampling that can reliably demonstrate the absence of infection. In addition, the testing of antibodies to FMDV NSPs gives no absolute guarantee of freedom from infection, as a significant proportion of vaccinated carrier animals may fail to demonstrate an anti-NSP response

(Mackay, 1998). Certain factors such as breed, management conditions and other pathogens could influence the specificity of the tests (Greiner & Gardner, 2000). Another important factor that may influence the discrimination capacity of these assays is the residual NSPs in commercial vaccines that may cause a rise in antibodies to the NSPs when animals had been vaccinated several times (Mackay *et al.*, 1998; Bergmann *et al.*, 2000). However, this test could probably be used on a herd basis when it has been fully validated according to an established and statistically valid sampling frame.

Based on the limited results presented in this paper it is not possible to recommend one single kit for use in regions where the SAT types predominate. Due to costs it is also not possible to use a combination of kits. More results are needed before a final recommendation can be made.

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EVALUATION OF THE LEVEL OF KNOWLEDGE OF ANIMAL HEALTH TECHNICIANS ABOUT PARASITE RECOGNITION AND CONTROL IN CATTLE

M J Sekokotla, CME McCrindle

Abstract

Animal Health Technicians(AHT) need sufficient knowledge about recognition and control of cattle parasites to perform veterinary extension effectively to cattle farmers .The state employs ATHs who qualified at different institutions including Technikon RSA, Fort Cox Agricultural College, Tompi Seleka Agricultural College, Fort Hare University, and the University of the Northwest and Taung College of Agriculture do Veterinary extension. The qualifications included two-year certificates, three-year diplomas, four-year diplomas, B Tech and B.Sc. Agric (Animal Health). The recognition and control of cattle parasites forms part of the curriculum in all of these qualifications. In addition, in-service training is done by state veterinarians.

Two groups of AHT were used. The first group (n=15) came from North-West Province (NWP) and the second group (n=29) from Limpopo Province (LP). They were all given the same evaluation before and after a short refresher course on the recognition and control of parasites in cattle. The extension was a refresher course and included the material required to do extension to the farmers for cattle parasite control.

As in a test performed to assess student knowledge at a tertiary institution, 50% was taken as an acceptable mark. The mean score before the course for AHT from NWP was 35.64%, which was not acceptable. Only one respondent was above 50%, the range was from 1.82% to 54.55%. After the course the mean improved significantly to 54.50%, with a range of 7.27% to 82.93%. This was still low, however a single respondent with a very low mark of 7.27% influenced the mean. Five of the respondents out of 15 were still below 50% on the second test.

It was found that none of the AHT from Limpopo Province passed with 50% or higher before the course and, although there was a significant improvement, only 17 of the 26 who wrote the second test (three did not write the second test), passed. The mean score before the course was 27.50% (range 7.32-43.90%) and after the course was 49.72% (19.51-68.29%). The level of knowledge of the AHT involved in this evaluation was not considered to be adequate, although AHT from NWP were higher than those from Limpopo Province and further in-service training was recommended.

These results were rather surprising, considering that a large amount of the routine veterinary extension to farmers is about tick and worm control. It was not anticipated that the base-line knowledge of AHT would be so very low, definitely this level of knowledge is unacceptable and should be addressed by the state veterinary services.

Key words: **Evaluation of Animal Heath Technicians, Veterinary Extension, State Veterinary Service Delivery.**

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SUMMARY

Animal Health Technicians (AHT) need sufficient knowledge about recognition and control of cattle parasites to perform veterinary extension effectively to cattle farmers .The state employs ATHs who qualified at different institutions including Technikon RSA, Fort Cox Agricultural College, Tompi Seleka Agricultural College, Fort Hare University, and the University of the Northwest and Taung College of Agriculture do Veterinary extension. The qualifications included two-year certificates, three-year diplomas, four-year diplomas, B Tech and B.Sc. Agric (Animal Health). The recognition and control of cattle parasites forms part of the curriculum in all of these qualifications. In addition, in-service training is done by state veterinarians.
Two groups of AHT were used. The first group (n=15) came from North-West Province (NWP) and the second group (n=29) from Limpopo Province (LP). They were all given the same evaluation before and after a short refresher course on the recognition and control of parasites in cattle. The extension was a refresher course and included the material required to do extension to the farmers for cattle parasite control.
As in a test performed to assess student knowledge at a tertiary institution, 50% was taken as an acceptable mark. The mean score before the course for AHT from NWP was 35.64%, which was not acceptable. Only one respondent was above 50%, the range was from 1.82% to 54.55%. After the course the mean improved significantly to 54.50%, with a range of 7.27% to 82.93%. This was still low, however a single respondent with a very low mark of 7.27% influenced the mean. Five of the respondents out of 15 were still below 50% on the second test.
It was found that none of the AHT from Limpopo Province passed with 50% or higher before the course and, although there was a significant improvement, only 17 of the 26 who wrote the second test (three did not write the second test), passed. The mean score before the course was 27.50% (range 7.32-43.90%) and after the course was 49.72% (19.51-68.29%).
The level of knowledge of the AHT involved in this evaluation was not considered to be adequate, although AHT from NWP were higher than those from Limpopo Province and further in-service training was recommended.
These results were rather surprising, considering that a large amount of the routine veterinary extension to farmers is about tick and worm control. It was not anticipated that the base-line knowledge of AHT would be so very low, definitely this level of knowledge is unacceptable and should be addressed by the state veterinary services.
Key words: Evaluation of Animal Heath Technicians, Veterinary Extension, State Veterinary Service Delivery.
Sekokotla MJ, McCrindle CME Evaluation of the level of knowledge of animal health technicians about parasite recognition and control in cattle *Proceedings of the 5th South African Society for Veterinary Epidermiology and Preventive Medicines, South Africa 14 August 2004*. SASASVEPM.

INTRODUCTION

Animal Health Technicians (AHT) need sufficient knowledge about recognition and control of cattle parasites to perform veterinary extension effectively to cattle farmers [2,7]. Part of the duties of state veterinary services is the surveillance and control of parasitic and contagious diseases of cattle [7]. Tick-borne diseases are of major economic importance in South Africa. Internal parasites of cattle, in particular tape worms causing cysticercosis, can play a role in veterinary public health as well as having an effect on the health of the cattle [3,8,9,10,11]. The state employs ATHs who qualified at different institutions including Technikon RSA, Fort Cox Agricultural College, Tompi Seleka Agricultural College, Fort Hare University, and the University of the Northwest and Taung College of Agriculture do Veterinary extension. The qualifications included two-year certificates, three-year diplomas, four-year diplomas, B Tech and B.Sc. Agric (Animal Health). The recognition and control of cattle parasites, forms part of the curriculum in all of these qualifications. In addition, in-

service training is done by state veterinarians. AHT in the state veterinary services are expected to do veterinary extension to farmers using the visit and training method [1,4,7].

MATERIALS AND METHODS

The knowledge and skills of two groups of AHTs were evaluated and they were trained in February 2000 and March 2000 respectively, during a one-day refresher course on the recognition and control of internal parasites.

The first group (n=15) came from North-West Province (NWP) and the second group (n=29) from Limpopo Province (LP). They were all given the same evaluation before and after the same short course. The extension was a refresher course and included the material required to do extension to the farmers for cattle tick and worm control. A questionnaire was used to evaluate the AHT before and after training was offered to them at a refresher course. Data was analysed using a spreadsheet .

RESULTS

As in a test performed to assess student knowledge at a tertiary institution, 50% was taken as an acceptable mark. The mean score before the course for AHT from NWP was 35.64%, which was not acceptable (Fig 1). Only one respondent was above 50%, the range was from 1.82% to 54.55%. After the course, the mean improved significantly to 54.50%, with a range of 7.27% to 82.93%. This was still low, however the single respondent who had a very mark of 7.27% was an outlier that lowered the mean. Five of the respondents out of 15 were still below 50% on the second test.

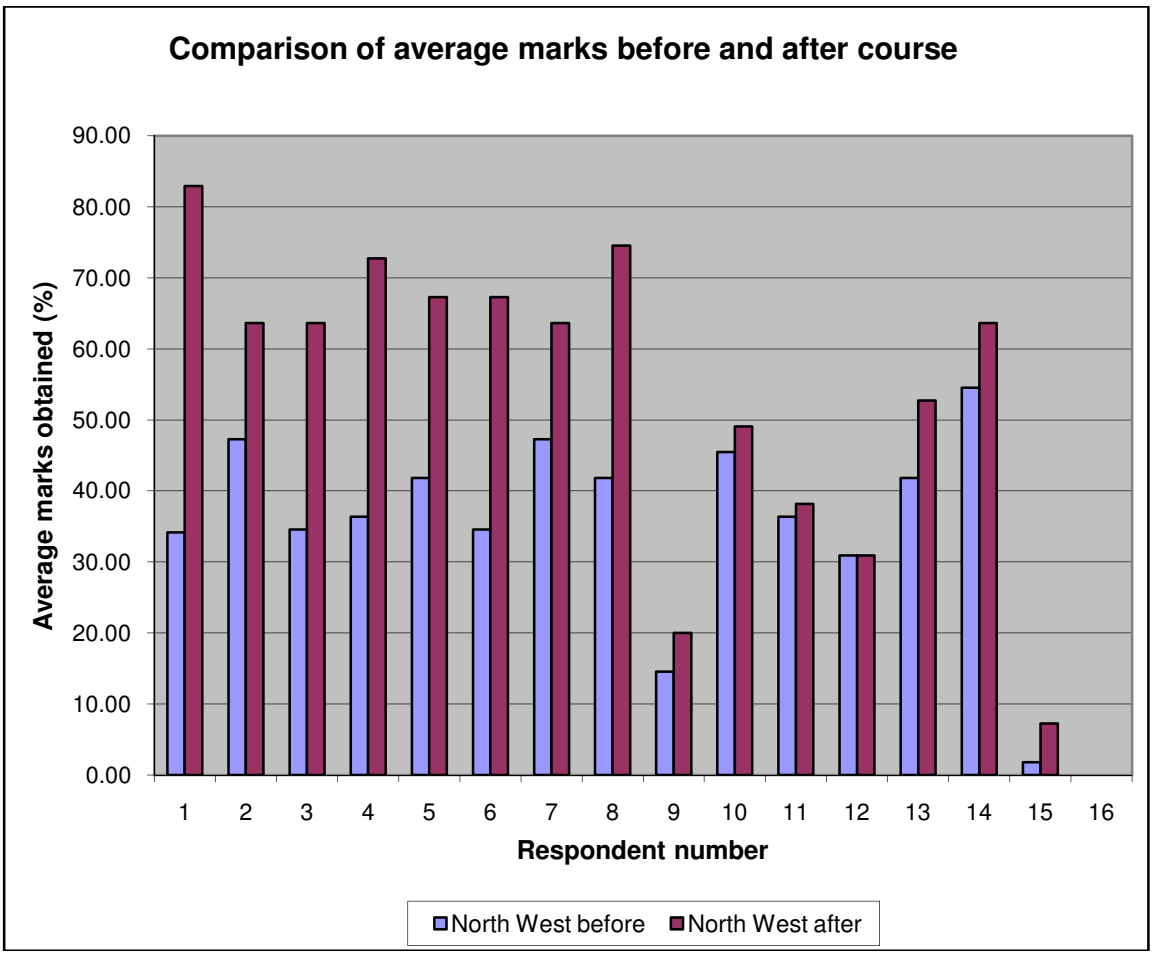

Fig 4.1 A comparison of the average marks obtained before and after a course on tick and worm control by AHT (n=15) in North-West Province

It was also found that with AHT from Limpopo Province, none of the AHT passed with 50% or higher before the course and, although there was a significant improvement, only 17 of the 26 who wrote the second test (three did not write the second test), passed (Fig 2). The average mark before the course was 27.50% (range 7.32-43.90%) and after the course was 49.72% (19.51-68.29%).

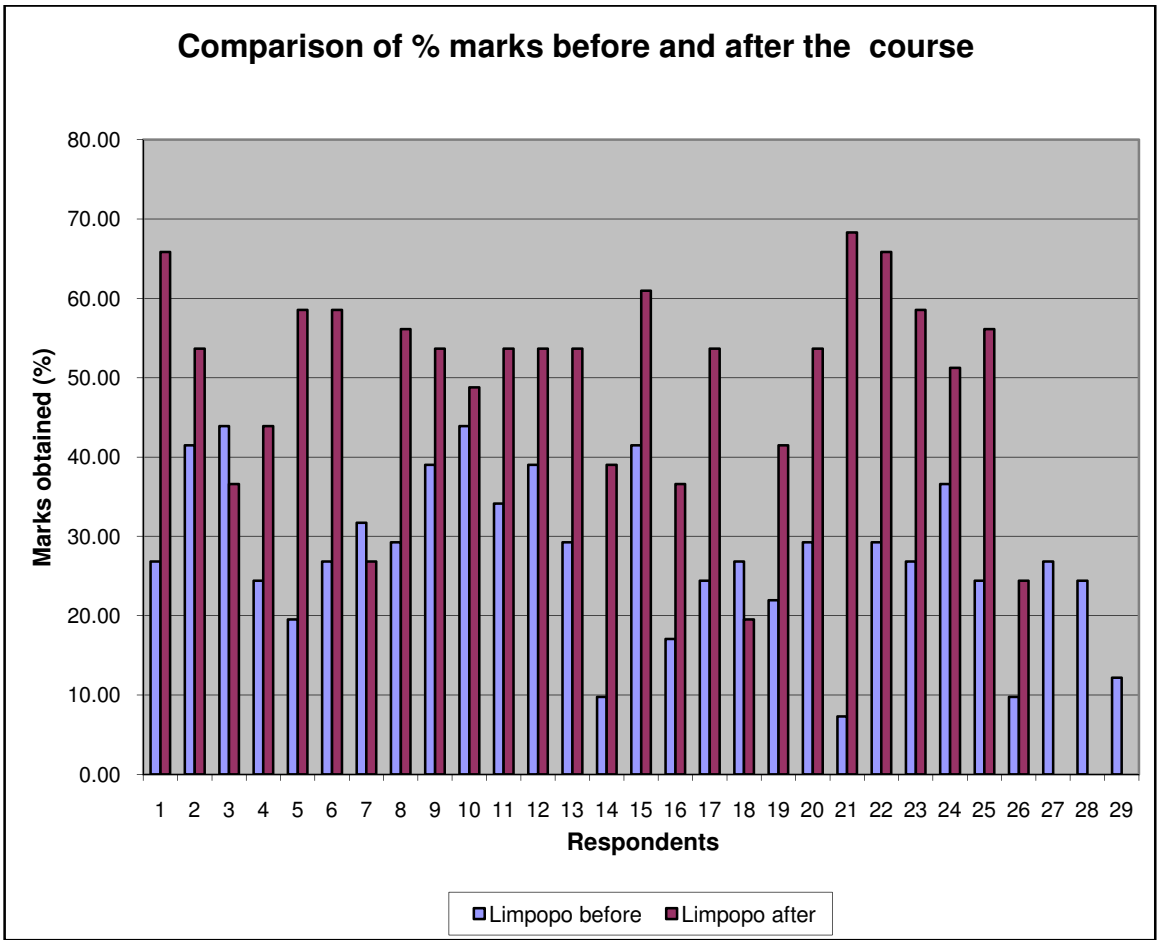

Fig 2 A comparison of the average marks obtained before and after a course on tick and worm control by AHT (n=29) in Limpopo Province.

It was not anticipated that the base-line knowledge of AHT would be so low, definitely this level of knowledge of this important aspect of parasite control is unacceptable and should be addressed by the state veterinary services.

CONCLUSION AND RECOMMENDATIONS

It is accepted that for extension to work, those presenting the extension should have a sufficient level of knowledge of the subject [4,6]. The course presented to the AHT's was supposed to be a refresher course and included the material required to do extension to the farmers to assist them with skills and knowledge required for parasite control. However the level of knowledge of the AHT involved in the study was not considered to be adequate (although NWP was higher than Limpopo Province). Further in-service training was required to bring the AHT up to the accepted

level. The results were rather surprising, considering that a large amount of the routine veterinary extension to farmers is about tick and worm control.

It is therefore recommended that frequent refresher courses be offered to state agricultural extension personnel so that their memories are refreshed on the dynamics of extension service delivery and technical knowledge on disease and parasite control.

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PRELIMINARY RESULTS ON A BOVINE TUBERCULOSIS OUTBREAK INVESTIGATION IN MPUMALANGA AND GAUTENG PROVINCES USING *MYCOBACTERIUM BOVIS* GENETIC STRAIN TYPING

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ABSTRACT

Mycobacterium bovis is the causative agent of tuberculosis in cattle, resulting in a greater loss to agriculture worldwide, as well as in a range of animal species including humans. This research focuses on the use of restriction fragment length polymorphism (RFLP) method to investigate the epidemiology of a bovine tuberculosis outbreak in the Middelburg district, Mpumalanga Province. In June 2003, a dispersal sale of Friesland cattle and calves was held in the Witbank district and TB infected cattle were sold onto at least 13 farms in Mpumalanga and Gauteng Provinces. In August 2003, the first diagnosis of the outbreak was made in a dairy cattle herd in which 5 out of 13 cattle purchased at the auction tested positive for bovine tuberculosis (BTB) during routine testing using the intradermal tuberculin test. Between August and December 2003, a further 12 farms were actively traced and *M. bovis* infection was confirmed on 10 farms. One of the farms had been diagnosed with BTB prior to the livestock auction. *M. bovis* was isolated from 84 out of 134 slaughtered, test positive cattle. No isolation was made from milk samples cultured from 17 cattle on 6 farms involved. Preliminary results on *IS*6110 RFLP typing of the *M. bovis* isolates from different farms provide an indication on the genetic relatedness amongst them.

INTRODUCTION

Bovine tuberculosis (BTB) is a chronic bacterial disease of animals and humans caused by the acid-fast, slow-growing, non-photochromogenic rod-shaped *M. bovis*. The disease has a worldwide distribution. In many countries, bovine tuberculosis is still a major infectious disease among cattle. Although cattle are considered to be the true host of *M. bovis,* the disease has been reported in a number of other domestic and wildlife populations (8). Bovine tuberculosis in South African wildlife was first reported in 1929 in greater kudu (*Tragelaphus strepsiceros*) and common duiker (*Sylvicapra grimmi*). Transmission of *M. bovis* infection to humans constitutes a public health problem but only few countries have documented data on the role of *M. bovis* in human tuberculosis epidemics (5,7). The prevalence of BTB is generally higher in dairy than in beef cattle. This may be due to closer confinement, longer life span and the stress demanded of greater productivity among dairy cattle (5). Restriction fragment length polymorphism is an internationally applied standardized method that is most frequently used in epidemiological investigations involving *Mycobacterium tuberculosis* as well as *M. bovis.* (12). The genetic marker mostly used in RFLP method is the insertion sequence (IS) element *IS*6110 which is present in several copies in *Mycobacterium tuberculosis* complex strains (6,9,10,11,12,14). It has the ability to move within the genome to different locations and chromosomal restriction fragments carrying this element are highly polymorphic (13). In recent studies, typing of *M. tuberculosis* complex strains by *IS*6110 RFLP has shown to be an excellent epidemiological tool for studying outbreaks, nosocomial infections, human immunodeficiency virus associated transmission, and the dissemination of multidrug resistance strains (1,3,4,6,11,13). In Europe and New Zealand the frequent occurrence of strains with only one to two copies of *IS*6110 has limited the value of this probe on its own for such strains. In South Africa a high diversity of *M. bovis* strains containing between one and five copies

of *IS*6110 have been observed, the latter of which provided a sufficient level of polymorphism to allow similar trace-back studies (15).
The aim of this study is to analyse and compare *M. bovis* strains isolated from different BTB infected cattle herds in the Middelburg district using *IS*6110 RFLP in the first step of characterisation.

MATERIALS AND METHODS

Tissue samples

Tissue samples from 134 slaughtered reactor cattle from 13 different farms linked to a dispersal sale of infected cattle were received by the Tuberculosis Laboratory of the Onderstepoort Veterinary Institute for confirmation of BTB. In table 1 animal identifications and corresponding laboratory (TB) numbers of *M. bovis* isolates from cattle originating from 6 farms are listed.

Bacterial isolation of *M. bovis*

Mycobacterium bovis was cultured from tissue samples submitted for routine diagnosis and processed as described previously (2).

Table 1. Identification of *M. bovis* isolates, cattle and farm of origin

M. bovis isolate No.	Animal identification	Farm
TB3912B	No.33	A
TB3924B	No.104	B
TB3925A	No.64	C
TB3927B	No.2	D
TB3932C	No.0003	E
TB3932K	No.9867	E
TB3940C	No.0203	E
TB3940D	No.0234	E
TB3969C	No.0142	E
TB3975B	No.9991	E
TB3976B	No.9946	E
TB3978E	No.0096	E
TB3979D	No.0022	E
TB3980D	No.9652	E
TB3980F	No.9838	E
TB3981B	No.0037	E
TB3981D	No.0101	E
TB3981F	No.0105	E
TB3983E	No.0125	E
TB4018	No.146	F

Restriction fragment length polymorphism analysis

Mycobacterial strains, culture media and conditions

M. bovis strains isolated from tissue samples of BTB infected cattle were subcultured on Lowenstein-Jensen media slopes supplemented with pyruvate and incubated at 37°C for up to 6 weeks or until sufficient growth was observed.

Chromosomal DNA extraction

DNA extraction was done using the Puregene DNA isolation kit (Roche) following the manufacturer's instructions with minor modifications. Overnight glycine-treated cells were heat-killed at 94°C for 10 minutes and allowed to cool down. 500µl of MSG extraction buffer (50g/L Monosodium Glutamic Acid; 6.06g/L Tris; 9.3g/L EDTA) were added to the culture and colonies were removed with an inoculation loop. Following centrifugation, 300µl of lysis and 5µl RNAse A (4mg/ml) solutions were added to the sample and incubated at 37°C for 30 minutes. Protein precipitation solution (100µl) was added and the sample was vortexed, centrifuged at maximum speed for 2 minutes, followed by precipitation of the aqueous phase with isopropanol. The resulting pellet was dissolved with TE buffer (10mM Tris-HCL; 1mM EDTA).

DNA restriction digest, Southern blotting, and hybridization

About 1.5µg of chromosomal DNA was digested with 0.5µl (15U/µl) of P*vu* II restriction enzyme at 37°C overnight. The resulting fragments were electrophoressed on a 0.8% agarose gel. Southern blotting was done by using the Hybond N^+ membrane in 20X standard saline citrate (175.3g/L NaCL; 88.2g/L Trisodium Citrate hyhrous) overnight. The *IS*6110 probe was synthesized and labeled using the DIG probe synthesis kit (Roche), hybridization and detection of the probe was done using the DIG-Easy Hyb granules and subsequent chemiluminescent detection according to the manufacturer's instructions (15).

RESULTS

Bacterial isolation of *M. bovis*

After up to 10 weeks of culture incubation, positive cultures were identified as *M. bovis* by Ziehl-Neelson staining and confirmed by polymerase chain reaction (PCR) technique.

Restriction fragment length polymorphism analysis

Of 134 cattle sampled from 13 different farms, *M. bovis* isolates from 20 cattle on 6 farms have been analyzed to date (see Table 1) using *IS*6110 RFLP typing. Three of the *M. bovis* strains (TB3912B; TB3924B; and TB3925A) were isolated from cattle purchased at the auction, the remaining isolates were made from in-contact cattle 3 – 6 months after the dispersal sale. On one of the recipient farms (farm E) bovine tuberculosis had been diagnosed and controlled by test-and-slaughter prior to this outbreak. The reactor rate following the second introduction of infected cattle was 23.5% of which 62% showed visible lesions at slaughter. *M. bovis* was confirmed by culture in 67 of 88 cattle slaughtered. Fifteen isolates characterised to date were found to be genetically identical to the isolates from 5 other farms (Fig. 1).

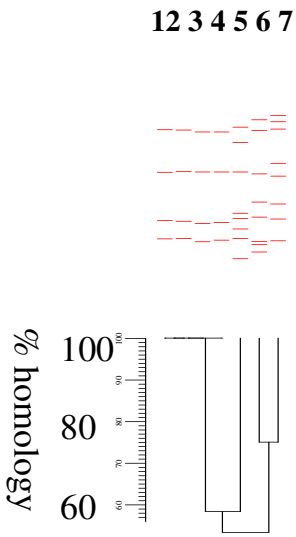

Figure 1: IS6110 RFLP patterns of *M. bovis* isolates. Lanes 1 – 4: BTB outbreak Middelburg district. Lanes 5 – 7: different BTB outbreaks in cattle in South Africa

DISCUSSION AND CONCLUSION

The epidemiology of infectious diseases has traditionally relied on observed patterns of occurrence to infer transmission. In this study, restriction fragment length polymorphism using the insertion sequence *IS*6110 was selected as the principle genetic fingerprinting method to study the spatial distribution of BTB following the dispersal sale of an infected cattle herd in the Middelburg district. At the sale the cattle herd was claimed to be free of BTB but the last intradermal tuberculin test on this herd had been conducted in 1996/1997.

Twenty *M. bovis* isolates from reactor cattle on 6 affected farms which had acquired cattle through the dispersal sale 3 – 6 months ago were found to show an identical *IS*6110 banding pattern representing 2 copies of *IS*6110 (Fig.1). Three of the isolates were derived from cattle originally purchased at the auction. These preliminary findings may support the outcome of the traditional outbreak investigation in suggesting the dispersal sale as the common source of the outbreak and also confirm BTB transmission from cattle acquired at the auction to subsequent in-contact animals in the new herds. The fact that the *M. bovis* strain involved possesses only 2 copies of *IS*6110, however, prompts the need for further probes and/or typing methods to be used to allow optimum discrimination.

Further analyses of additional *M. bovis* isolates from the remaining herds traced back as recipients of potentially infected cattle will help to determine the extent of this BTB outbreak. Genotyping can provide useful information in the case of farm E in which BTB had been reported prior to the dispersal sale. Although to date no indication of concurrent BTB outbreaks have been found among 15 isolates from farm E there is a possibility that cattle infected during the previous outbreak (from an epidemiologically unrelated source) have only been detected and slaughtered as part of this investigation.

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AFRICAN HORSE SICKNESS: OVERVIEW OF THE OUTBREAK IN THE WESTERN CAPE, 2004

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ABSTRACT:

African Horse Sickness (AHS) is a controlled disease in South Africa. For the purpose of control, the control area in the Western Cape Province is divided into three zones, the protection, surveillance and free zone. The location of the free zone was based on the historical absence of the disease and not the absence of vectors. (Lord et al., 2002)

In the 2½ years from June 2001 until December 2003 a total of 487 horses (149 Thoroughbreds, 111 Arabians and 52 other) were exported from the free zone to various countries. South Africa's export status can only be maintained by strict movement controls to-and-from the AHS control areas. These controls are aimed at the prevention of introduction of AHS into the surveillance or free zones since an outbreak anywhere in these zones can lead to a 2 year suspension of horse exports.

During the period 21 to 23 February 2004, four Percheron horses died acutely at the Elsenburg agricultural college in the Stellenbosch district. African Horse Sickness was subsequently diagnosed by virus isolation at the Onderstepoort Veterinary Institute. An intensive epidemiological investigation revealed several other cases in the surrounding areas, all occurring within the surveillance zone.

AHS outbreaks are rare occurrences in the Western Cape. During the 1999 outbreak (the first in many decades in the area), 32 horses died in the period 21 March to 17 May 1999. What is disturbing about the 2004 outbreak is that it occurred only 5 years after the previous outbreak, and then also before any cases had been reported from the rest of the country.

This paper will present an overview of the 2004 outbreak and will discuss the control measures implemented as well as the rationale behind them.

INTRODUCTION

The Western Cape Province hosts the only African Horse Sickness (AHS) free area in the country from where horses are accepted for export to the rest of the world. The free zone comprises a small area within the Cape Town metropolitan area.

From 2001 until 2003 a total of 487 horses (149 Thoroughbreds, 111 Arabians and 52 other) were exported from the free zone to many countries (including the United Arab Emirates, European Union, Singapore, Malaysia, Mauritius, Hong Kong and Bahrain). (Dunn, 2003) The exact income from these exports are unknown, but is estimated to be worth at least R65 million annually to the industry.

The benefits of maintaining a free zone cannot only be measured by the exports, but should take into account that these measures also allow foreign horses to enter the country and be able to return to their point of origin. Shuttle stallions can consequently be imported for a season to improve

bloodstock in South Africa and are able to return to their country of origin fairly easily. The maintenance of the free zone has also for the first time allowed top South African race horses to compete with great success in racing events abroad. It is therefore understandable why the Directorate of Veterinary Services did everything possible to contain the recent outbreak as quickly and effectively as possible.

DISEASE DESCRIPTION

African Horse Sickness (AHS) is an infectious, non-contagious disease of equines, which can manifest as a peracute, acute, subacute or mild disease. The disease is endemic to the African continent and is caused by an orbivirus that is transmitted by *Culicoides spp.* midges. Nine serotypes of the virus have been identified. Mortality rates can be as high as 95% in horses, while donkeys and mules rarely show clinical signs. (Coetzer and Erasmus, 1994)

Donkeys can have a persistent viraemia for at least 12 days. They must be considered a potential source of virus for midges and can act as "silent reservoirs", although it is very unlikely that they are long-term reservoirs for the virus. (Hamblin et al., 1998)

Zebra do not show any clinical signs of disease and can serve as a reservoir of infection. Virus can be isolated up to 40 days after infection from blood and up to 48 days after infection from the spleen. (Barnard et al., 1994) The capability of zebra to maintain AHS virus is clearly illustrated by the persistence of infections during every month of the year, with a peak period in winter. This peak is attributed to the presence of large numbers of susceptible foals in the presence of active *Culicoides* species. (Barnard, 1993)

Culicoides midges are abundant in the Boland area (surveillance zone) virtually throughout the year. The midges need an infected horse as a source of virus, to enable transmission of the disease to susceptible horses – usually at a rapid rate, irrespective of farm boundaries.

African Horse Sickness normally spreads from the northern and eastern parts of South Africa to the interior in late summer and autumn. Clinical cases are usually only expected from March onwards. (Coetzer and Erasmus, 1994) The southward spread of the disease varies in distance depending on the time of first appearance, the number of susceptible equids and the favourability of climatic conditions for breeding of the insect vector. The movement of actively infected horses can obviously facilitate the spread. (In 1999, the infection was spread to the Western Cape (Stellenbosch area) by the introduction of an infected animal from the Free State Province.) This southward spread is terminated abruptly by the first heavy frost in May. (Bosman et al., 1995)

START OF THE 2004 OUTBREAK

The first indication that there was an outbreak of African Horse Sickness in the Surveillance Zone was when four Percheron horses died within a few days of each other from 21st to 23rd February at the Elsenburg Agricultural College, 10 kilometres north of Stellenbosch, and about 38 kilometres east of the Horse Export Quarantine Facility at Kenilworth in Cape Town. In retrospect it would appear that the first (or indicator) case occurred on the 31st January 2004, when a Percheron horse died of what was initially diagnosed as annual rye grass toxicity. Samples taken from this initial death were then retested and came back positive for AHS. Outbreaks of annual rye grass toxicity are known to occur during late summer and early autumn in the area, a fact that contributed to the initial confusion.

After the second case of AHS at Elsenburg, deaths occurred intermittently in the surveillance zone up until the 28th of March, on which date the last confirmed death occurred. A total of 16 horses died in this 10-week period. (See Figure 1 for geographical distribution.) Several press releases were issued to keep the public and horse industry informed. Targeted preventative actions were implemented as the disease progressed in order to limit the spread of the infection as far as possible and to avoid compromising the disease-free status of the Horse Export Quarantine Station in the AHS Free Zone.

The surveillance area acts as an early warning or sentinel system for the free area and horses are therefore normally not vaccinated against the disease in accordance with the export protocol negotiated with the European Commission. The early detection of the disease in this area proved this measure to be effective, but at the unfortunate cost of the loss of 5 very expensive Percheron Stud horses. This breed is apparently twice as susceptible as other horses to AHS, and to compound the matter, were unvaccinated, unstabled at night and pastured in a vlei area. The infection spread mostly in a northwesterly direction, with the closest case to the free zone occurring a distance of 24 kilometers from its eastern boundary. The integrity of the Free zone was consequently never compromised at any stage of the outbreak.

SPECIFIC ACTIONS TAKEN AND PARTIES INVOLVED IN CONTROL

On the 26th of February 2004 an emergency contingency planning meeting was called at Elsenburg with all the relevant role players. This included local equine specialist veterinarians, personnel from the Disaster management team of the Western Cape and staff of the provincial Directorate of Veterinary Services. The following actions for implementation were agreed upon:

(i) A 30-kilometre buffer zone was created using the first diagnosed case at Elsenburg as the epicentre, even though it was highly unlikely that it was the causal case since there had been absolutely no movement of horses to the farm for at least the previous 2 months.

(ii) Animal Health Technicians (AHT's) were called in from neighbouring State Veterinary areas to assist with surveillance on equine holdings in the area. Ten officials were deployed in a 30 km zone around the epicenter and issued with questionnaires which included a census of the horses, information on movements, vaccination status, clinical evidence disease or unexplained deaths of horses. Within a period of about 3 weeks 1616 farms had been visited, representing some 4491 horses of which approximately 33% had not been vaccinated at all or at least not within the last year.

(iii) The Department of Agriculture of the Western Cape immediately made 1 400 doses of AHS vaccine available free of charge to private veterinarians (PV's), who were requested to agree on charging a reasonable fee to all their clients. The AHT's assisted in vaccinating in resource-poor communities and no charge was levied in these instances.

(iv) An immediate total embargo was placed on all horse movements within the whole of the Surveillance and Free zones and the Minister of Agriculture issued the first of many Press Releases on 27/02/04.

(v) The Provincial and Municipal Traffic Departments within the Disaster Management team played a vital role in assisting the Directorate by monitoring and restricting movements of horses. Apart from the Press releases, several interviews were done with the national television stations and regular updates were broadcasted over the local radio stations throughout the duration of the outbreak. A 15-minute documentary was also produced for Teletrack on Channel 34 for Digital Satellite Television (DSTV). This program highlights important events, which affect the Race Horse community, and has wide coverage in the racing fraternity. Situation reports were also compiled intermittently to keep the relevant authorities and private veterinarians informed of the evolvement of the outbreak, progress with disease control measures and the status of the quarantine restrictions.

The complete ban on horse movements within the entire Surveillance zone appeared to have had a significant economical impact on the race horse community and associated businesses. The Department of Agriculture called another stakeholder meeting on Sunday 29th February in order to review the situation in the light of the apparent localization of the outbreak in the Stellenbosch area, which is approximately 35 kilometers away from the Racing Stables at Milnerton, the Kenilworth race track and the horse Export Quarantine Station. The Minister of Agriculture chaired the meeting, which was attended by representatives from Gold Circle Racing (GCR), Thoroughbred Breeders Association (TBA), trainers, the Directorate Veterinary Services and the media.

Following intensive discussions it was agreed to allow restricted movement of race horses between the racing stables situated in low risk areas, to Kenilworth, provided they met certain stringent conditions. These included Veterinary health certification, treatment of the horses and horse boxes with insecticides, vaccination status, stabling overnight from 2 hours before sunset until 2 hours after sunrise, restricted movement times only between 2 hours after sunrise until 2 hours before sunset and issuing of Red Cross permits for each movement.

Disaster Management team meetings were held 2-3 times per week initially at Elsenburg to keep the Provincial and Municipal traffic authorities informed on progress and the disease status. Only horses with red cross permits issued by the State Veterinarian Boland were allowed to move. Anyone caught without the correct documentation was instructed to return to point of origin immediately. This system worked extremely well and was implemented very efficiently by the traffic officers on duty.

Due to the apparent limited spread of the virus and after comparing the risk involved with the negative effects on the industry, the quarantine in most of the surveillance zone was lifted on 11 March 2004. However, the Stellenbosch and Somerset West magisterial districts, which were considered to harbour the primary focus of infection, 11 properties in the Bottelary district and a single farm in the Franschhoek district, remained under strict quarantine.

An additional area was placed under quarantine after a horse died peracutely on a plot 15 kilometres south of Malmesbury and approximately 32 kilometres northwest of Elsenburg on Sunday 28th March. A diagnosis of AHS was confirmed and all the horses in this area were vaccinated. Three weeks later another horse became ill in this area and subsequently tested positive for AHS, but it recovered after a few weeks. Since no movements of horses by road occurred in the vicinity, it was concluded that these cases occurred as a result of natural spread of the disease.

After the last confirmed death on 28th March the quarantine in the designated areas (with the exception of Malmesbury) was maintained for a further period of 7 weeks before the quarantine was lifted on 17th May 2004. This enabled sufficient time for any further outbreaks (clinical or deaths) to occur and allowed time to process all the serology results from the numerous horses sampled during the initial survey in the 30-kilometre zone.

With no further cases occurring in the Malmesbury district, the quarantine was lifted on the 21st June.

SOURCE OF THE INFECTION

The exact source of the infection is as yet not known. The virus involved in the outbreak was typed as AHS serotype 1, which does not occur commonly in South Africa. What was even more puzzling was the fact that horse sickness outbreaks had not yet been reported from the Infected Zone in the rest of the country. It appeared to be too early in the season for AHS and this was responsible for the delay in diagnosing the initial death as also being due to AHS. Once the causal

virus had been typed, a clearer picture emerged. In 2002 samples sent from Springbok tested positive for AHS type 1. These samples originated from Namibia. It is thus suspected that there may have been an illegal movement of a horse incubating the disease from somewhere in Namibia into the Surveillance zone in close proximity to Elsenburg.

CONSEQUENCES OF THE OUTBREAK

Due to immediate disease control actions taken, especially the rapid vaccination campaign and strict movement controls, only 16 horses died and about the same number showed clinical signs but recovered in the Stellenbosch district where the main focus of the disease was situated. Two other isolated deaths occurred outside this area but were linked to the main core as they were all confirmed as the same serotype of the AHS virus (serotype 1). The number of deaths is however relatively low, compared to the morbidity and mortality rates in Gauteng, where 75 cases already developed between February and March 2004.

The impact of the outbreak in the Western Cape on the racing community and other equestrian events was immense. Several race meetings and numerous large shows were cancelled as all horse movement ceased completely. Most importantly exports were placed under embargo and the whole export industry came to an immediate standstill, which represents a loss of millions of Rand in foreign exchange.

FUTURE FOR THE INDUSTRY

The similar outbreak in the area 5 years previously, with a mortality of 32 horses and an enforced 2-year ban on exports by the European Union, resulted in a substantial loss in foreign income for the racing industry in South Africa and breeding animals for local owners.

The AHS free status of the Western Cape was again compromised with the recent outbreak, but the deaths were far less, possibly due to stringent and immediate measures taken to control the disease.

The European Commission, being informed of the measures taken, agreed to allow South Africa to submit evidence to consider lifting of the ban before the compulsory period of two years provided for in the current protocol. A submission will therefore be made to the European Commission as soon as the Director of Veterinary Services is satisfied that the disease situation has stabilised sufficiently to render the required sanitary guarantees to the European Commission for the export of disease-free horses.

The movement restrictions on horses within the AHS control zone of the province, however, place an additional burden on all interest groups involved in the horse industry. It is therefore accepted that alternative options should be investigated to facilitate exports while at the same time not inhibiting the needs of other non-export interest groups within the equestrian society. Any alternative option would however, imply a change in the international standards for the trade in horses recommended by the World Organisation for Animal Health (OIE) and will therefore have to be negotiated with both the OIE and the European Commission.

Figure 1 : African Horse Sickness Free Area and Proximity of Positive Cases

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INVESTIGATION OF CONSTRAINTS TO REPORTING OF CATTLE MORTALITIES

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SUMMARY

The aim of the study was to assess the constraints to accurate reporting of cattle mortality in Odi district, North West Province. The method that was followed in this study was based on participatory action research. The cattle owning community of Odi district participated at every phase. They were the ones who first spoke to veterinary services about ways to decrease the diseases and mortalities of their cattle. Out of this came a questionnaire so that the actual facts could be determined.
A total number of 60 farmers were randomly selected from 12 villages. One farmer pulled out and we were left with 59 farmers. Since the area of study was communal, most farmers were men (n=55) and only four of them were female.
It was suspected that there was a communication problem and this was proven, as 23 farmers were not even aware that mortalities have to be reported by law. The real problem was that causes of death were not being diagnosed because farmers were not aware that a necropsy could give information on the causes of death. Farmers were keen to receive training in necropsy techniques so as to be able to discuss the cause of death of cattle with the state veterinarian.

INTRODUCTION

Most people in Odi district live in rural areas and depend on agriculture for their livelihood. Livestock may also be a form of saving, which can be cashed in through the sale of animals when needed. Diseases of livestock can cause major losses, both to livestock owners and the country as a whole (Cameroon, 1982). Commercial cattle farmers earned R2791 million from slaughtering of cattle and calves in 1996 (National Department of Agriculture, 1996). Farmers lose a lot of money when their cattle die. From the June edition of the Farmer's Weekly (2000), the slaughter price of beef was R9.00/kg and the weaner live-weight price was R5/kg (Janovsky, 2000). The weakest link in the reporting chain is usually the livestock owner, who may not recognise the disease, or may fail to report it for other reasons (Cameroon, 1982).
A high mortality rate in a herd has a depressing effect on the financial results of the farming enterprise (Carstens, 1971; Shoo *et al*., 1991)). Although it is not possible to eliminate stock losses completely, it is a controllable factor that can be minimized by means of an effective disease control programme and good management. According to Ristic and McIntyre (1981), it is important to know the level of mortality of cattle in order to be able to know the major diseases which cause mortality and be able to prevent and control causative agents. This paper shows the results of a survey of cattle owners (n=59) in Odi District of North West Province of their opinions on the reasons for lack of communication with state veterinary services about mortalities in their cattle.

MATERIALS AND METHODS

Two-stage cluster sampling (Thrusfield, 1995) was done where villages were the primary units and the farmers were the secondary units. Twelve villages were randomly selected from the 55 villages in Odi District. Non-random selection criteria (purposive selection) was done according to Dagartz and Hill (1996) to select farmers using the following criteria:
Only farmers with cattle were selected
Farmers had to show a willingness to participate
A minimum of 2 farmers were selected from each of the 12 villages.

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Twelve villages were selected. A Geographical Positioning System (GPS) was used to locate the farms. The location of the farms and the names of the villages are shown in Table 1.

Table 1: GPS location of farms and number of participants per village

Name of village	No	Name of farm	Farm no	Geographical location
Sephai	6	Roodekuil	179 JQ	27° 50′ 00 E, 25 15 00 S
Bethanie	8	Bethanie	405 JQ	27° 37′ 00 E, 25 34 00 S
Rietgat	3	Rietgat	224 JQ	27° 50′ 00 E, 25 25 00 S
Modikwe	4	Waaikraal	396 JQ	27° 35′ 00 E, 25 33 00 S
Bapong	7	Modderspruit	458 JQ	27° 41′ 00 E, 25 43 00 S
Boschpoort	3	Boschpoort	288 JQ	27° 29′ 00 E, 25 31 00 S
Winterveld	6	Winterveld	101 JR	27° 59′ 00 E, 25 24 00 S
Rabokala	5	Oskraal	248 JQ	27° 55′ 00 E, 25 30 00 S
Hebron	5	Mamogales-kraal	258 JR	28°00′ 00 E, 25 36 00 S
Legonyane	6	Elandsfontein	180 JQ	27° 47′ 00 E, 25 17 00 S
Maboloka	5	Klipgat	243 JQ	27° 53′ 00 E, 25 26 00 S
Mmakau	2	Elandsfontein	432 JQ	25° 27′ 00 E, 25 36 00 S

Most of the people in Odi district, which was formerly part of the homeland known as Bophuthatswana, speak the Setswana language, although other languages like Zulu, Xitsonga, Xhosa, Northern Sotho, Southern Sotho, Venda also exist in this area. It was estimated by Malan and Hatting (1975) that 50% of the population live in Mabopane and Ga-Rankuwa, which are urban areas, and the rest of the population live in peri-urban and rural areas. Most of the rural areas are poorly developed and lack infrastructure such as electricity, telecommunications, transport and water (Pistorius and Gumbi, 1997). The communities still maintain their traditional heritage, norms and values, which can be noticed from the way in which people co-operate with the chief and the headman (McCrindle *et al.*, 1994).

A total of 60 farmers were selected for structured interviews. A structured interview is a structured procedure with scientific purpose by means of which the respondent, through a series of questions, is induced to give verbal information (Pfeiffer, 1996; Simpson and Wright, 1988). The number of farmers selected from each village is shown in Table 1. One of the farmers at Mmakau could no longer participate and was eliminated from the trial. Data obtained from the questionnaire was typed into the software programme Microsoft Excel ® and analysed using simple observational (qualitative) statistical methods. Results are reflected as Tables, Histograms and Pie charts (Armstrong et al, 1992).

RESULTS AND DISCUSSION

The fact that only four women were involved may be due to the fact that women are not interested in large animals like cattle, they are more interested in small animals like goats and sheep and chickens. In the communal system, there is still complete dominance by male farmers. In comparison with Nthakheni (1993) where 14% (n=57) of the female farmers were widows, in this study only one person (1,69%) was a widow.

The ages of farmers ranged from 20 to 89 years old with most farmers in the range of 50 and 69 years (Fig 4.1). This is probably because young people are not interested in farming and the old ones take up farming after taking packages from their work place and buy cattle as a form of security and investment of savings. The older the farmer, the more forgetful they become. They do not keep records and tire easily and this impacts on their management, which then becomes poor and affects the productivity of their animals. Because of their advanced age, sometimes they cannot walk long distances to Service Centres to report sick and dead animals. Fig 1 shows the age distribution of the farmers. This agrees with the findings of other authors (Mokantla et al, 2004; McCrindle, 1997)

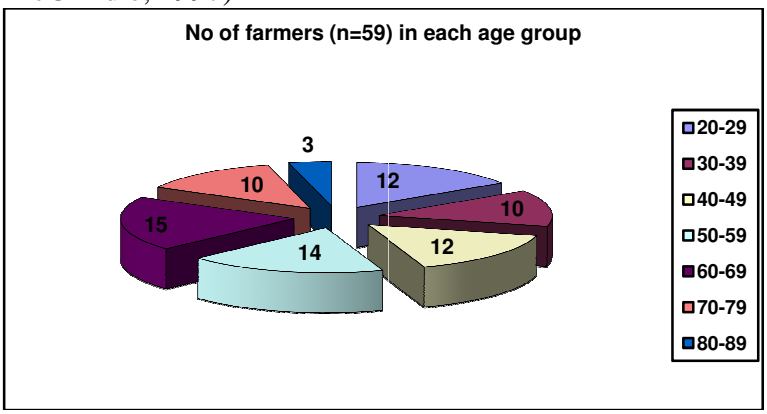

Fig 1: Ages of farmers in years (n=59)

The education level of the respondents varied, 21 of them (35%) went to high school, 19 (31%) of them went as far as primary school, 8 (16%) of them completed matric, 5 (8%) went to a tertiary institution and only 6 of them (10%) did not attend school at all. In general, cattle owners in communal areas are more prosperous and usually better educated than small-stock farmers as they require capital to start with cattle. Respondent`s main source of income was from full-time farming (n=25), pension (n=20) and salary of family member (n=20). Only four had other businesses. Cattle were mainly cared for by the owners (n= 41,69%), while 17% (n= 10) were looked often by herdsmen, 12% (n=7) by children and only 2% (n=1) by the wife of the owner. More than half of the participants looked after their own animals as they were not working or were on pension. Those who were working employed herdsmen to look after their animals. These herdsmen were also responsible for taking the cattle for vaccination during campaigns and reporting any sick cattle to the owner. Sometimes sick or dead animals are not reported in time to the state veterinarian as the owners work far from home and only see the animals over the weekend. Other members of the family also look after the animals.

Table 2: Gender and age distribution of cattle in the survey - census numbers and data obtained from questionnaires compared

Age/ gender	Number*	Actual number**
Bulls	38	45
Cows	686	700
Heifers	475	489
Bullocks	97	105

Oxen	46	47
Calves	357	376
Total	1699	1762

* Number estimated by farmers during the interview
** Number counted by AHT during census
The gender and age distribution and total number of cattle owned by communal cattle farmers in the survey is shown in Table 2. In surveying communal cattle owners, the "lie factor" has to be taken into account and it is advisable to also count the cattle. The actual number of cattle counted by the AHT as well as the number given by the cattle farmers is recorded. This difference may be due to the age of the farmers (they are forgetful), a low level of numeracy, lack of record-keeping, or a deliberate attempt to mislead (it is not always culturally acceptable to ask a person how many cattle they own and some fear being reported to the receiver of revenue).The distribution by breed is shown in Fig 2.

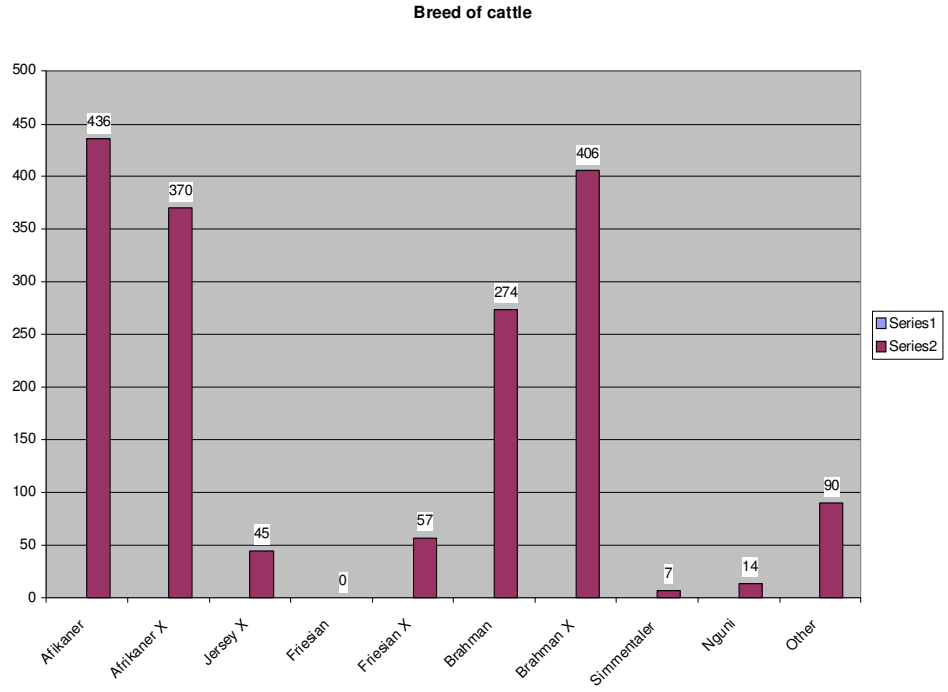

Fig 2 Distribution of cattle by breed

Communal farmers in Odi prefer a resistant type of breed that does not get sick or die easily because they farm extensively. The Afrikander and Brahman breeds are resistant to most diseases as well as ticks, that is why they were the preferred breeds. The cross-bred Afrikander/ Brahman breed has the advantage that calves grow faster and have a better slaughter percentage than purebred Afrikander cattle.
According to the results of interviews with farmers over a period of 12 months (from September 2000 to September 2001), the total percentage of cattle that died was 4,47% (n=76). The accepted percentage is 5%. The total percentage of 4.31% in this study was comparable to that of 4.91%, recorded by Mokantla (2003). The age and gender distribution is shown in Table 3.

Table 3: Percentage mortalities Sept 2000-Sept 2001 (Total n=1762)

Age/gender	Number of cattle	Number of cattle that died	% Mortality
Bulls	45	2	4.44
Cows	700	33	4.71
Heifers	489	13	2.65
Bullocks	105	2	1.90
Oxen	47	0	0
Calves	376	26	6.91
Total	1762	76	4.31

It may be noticed that the highest mortality is in the calves. Adult cows and bulls also have a relatively high mortality, probably because they are more easily stressed by lack of food in the winter than non-breeding animals (oxen, heifers and bullocks) and therefore their disease resistance is lower. The mortality resulting from infectious diseases is shown in Table 4.

Table 4: Infectious causes of mortality in cattle that died (n=11) during 2001 (Total n=1762)

Diseases	Number of cattle that died
Lumpyskin disease	6
Heartwater	2
Sweating sickness	1
Blackquarter	1
Anaplasmosis	1

The mortality of cattle from non-infectious causes is shown in Table 5 below. It should be noted that the cause of death was unknown in more than half (n=43) of the total deaths recorded (n=76).

Table 5: Non infectious causes of mortality (n=65) in 2001

Causes of death	Cattle mortality (n=)
Unknown	43
Dystocia	10
Plastic bags	6
Diarrhoea	2
Hairball	1
Snake bite	1
Fractured leg	1
Motor vehicle accident	1

Farmers (n=59) were also asked what they did with the carcasses of animals that died and their responses are shown below in Table 6.

Table 6: The fate of meat from dead animals

Reasons	Always (n-59)	Never (n=59)
People eat them	3	56

Dogs eat them	44	15
They rot away in the veld	22	37
Bury them	26	33
Burn them	21	38
People sell the meat	3	56

Proper disposal of carcasses is important both to prevent livestock disease transmission, and to protect air and water quality. The chosen method must be environmental friendly. The carcass must be disposed off as soon as possible.
Farmers were asked reasons why they failed to report cattle deaths to the State Veterinarian, AHT or Extension Officer. Deaths are only reported when the AHT routinely visits the farms in his ward. Their responses are ranked in order of frequency in Table 7.
Possible constraints for not reporting diseases were listed in the questionnaire and farmers were allowed to give more than one reason and these were rated from always, sometimes to never. The most important constraint seen from Table 4.7 was of not having any idea that they should report the dead animals to the State Veterinarian, AHT and Extension Officers. It is important to report dead animals to the state to be able to identify the disease and control it especially in cases of infectious diseases that spread quickly and causes economic losses to the farmers. Almost a third of the participants did not know that they should report dead cattle.
Communication has a vital role to play in any agricultural and rural development situation. Ideally the flow of information between the farmer, extension and research should be developed to an optimum degree. If the potential for improvement is to be realized, lack of communication must be addressed. From the questionnaire it was found that lack of communication, that is, having no cellular phone and no telephone at home were not major constraints to reporting mortalities. Veterinarians and technicians should always be available for farmers to report dead animals. However, the possible constraint of veterinarian not being in office and what can the vet do, were not a major reason for not reporting.
Transport can be an impeding factor in reporting diseases and mortalities. Most areas in Odi are rural and farmers are old and do not have access to transport. Some of the service centres are very far from them and the only mode of transport that they use are busses and they are not there most of the time because they have a time schedule, which is not sometime very convenient for them (Stewart, 1997).
Odi district consists of villages and most farmers work in surrounding towns like Pretoria, Johannesburg and Brits. The animal owners who are decision-makers regarding animal keeping and husbandry are absent during the weekdays and this leaves the herdsman stranded with decisions to make during this period (Letsoalo et al., 2000).

Table 4.8: Ranking of constraints in order of importance

Constraints	N=	Rank order
No idea of reporting (a)	23	1
Not interested (b)	13	2
No telephone at home (c)	11	3
No cell-phone (d)	10	4
No transport (e)	10	4
What can the Vet do? (f)	9	5
Not his business (g)	7	6
Carcass is eaten (h)	5	7
Vet is not in office (i)	3	8
I work in town (j)	2	9

Constraints (a), (b), (f) and (g) can be addressed by motivating farmers through extension that explains the benefits for them in knowing the causes of diseases. Constraints (c), (d), (e), (h) and (i) could possibly be addressed by the farmer

being trained to perform a necropsy when he finds the carcass and reporting back to the veterinarian who can give some idea of the possible causes of death and what to do about it. This also addresses the time constraint that the cadaver decays or is eaten before the veterinarian can get there.

CONCLUSIONS

After the questionnaire was statically evaluated, a participatory strategy was followed to assess the way forward. Farmer's opinions were gathered and it was decided to prepare appropriate extension material and hold an information day to increase the knowledge of causes of death of cattle. It was found that 84.75% of farmers were in favour of learning how to do a necropsy.

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THE ABILITY OF *Escherichia coli* IN RIVER WATER TO SURVIVE CHLORINATION TREATMENT

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SUMMARY

There is increasing concern about the ability of landowners and smaller local authorities to treat raw water obtained from rivers downstream from dense settlements. Should chlorine resistance be present to any significant degree in organisms occurring in free-flowing waters, this will imply that the ability to disinfect raw water from such polluted rivers is seriously impaired. The aim of this study was to establish the possible presence of organisms resistant to chlorine in the Plankenbrug River below a dense settlement with failing sanitation as indicator of environmental hazard.

E. coli organisms were isolated from river water by standard methods. A range of chlorine concentrations was used (0,1 - 0,6 mg/l total chlorine) as well as unchlorinated control. Survival was assessed at 30 minutes and 90 minutes after chlorine was added to the organisms.

After 30 minutes *E. coli* organisms survived up to the highest concentration employed, except for those exposed to 0,5 mg/l total chlorine. After 90 minutes contact time, *E. coli* organisms still survived up to 0,4 mg/l total chlorine.

If a disinfection system utilising 30 minutes' contact time is used to disinfect water from this river, then chlorine resistance will develop. The fact that organisms still survived up to the level just below optimal after 90 minutes of contact time, strengthens this conclusion. Water from the Plankenbrug River is therefore not suitable for disinfection with the ordinary methods available to small-scale users without increasing either the chlorine concentration and/or the contact time. This has implications for human health as well as for agriculture since irrigation water with a high chlorine concentration is phytotoxic to plants and affects animal health.

INTRODUCTION

Chlorination of drinking water has been one of the most effective public health measures ever undertaken (Bull, Birnbaum, Cantor, Rose, Butterworth, Pegram & Tuomisto, 1995). Chlorine has been successfully used for the control of waterborne infectious diseases for nearly a century. There are a number of alternatives to chlorination that are in active use in many parts of the world, but the risks associated with their by-products are even less well established than for chlorination (Bull *et al.* 1995). Moreover, the use of these alternatives vary in their effectiveness and some require greater sophistication in their application. This can mean less protection to public health as a result of inappropriate application and control.

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Sterilization is the complete elimination or destruction of all forms of microbial life (Rutala & Weber, 1997; Ayliffe, Coates & Hoffman, 1993) and is not achievable in the process of making

water potable. Disinfection, on the other hand, describes a process that reduces or eliminates many or all pathogenic micro-organisms with the exception of bacterial endospores (Rutala *et al.* 1997; Ayliffe *et al.* 1993). This process reduces the number of micro-organisms to a level that is not harmful to health (Ayliffe *et al.* 1993). Agents that kill all micro-organisms with the exception of bacterial endospores after exposure times shorter than those needed for sterilization (i.e. <45 minutes) are called high-level disinfectants. Intermediate level disinfectants may be cidal for tubercle bacilli, most viruses and fungi, but do not necessarily kill bacterial endospores (Rutala *et al.* 1997). Low-level disinfectants kill most vegetative bacteria, some fungi and some viruses within short exposure times (i.e. <10 minutes) (Rutala *et al.* 1997). An antiseptic is an agent that prevents or arrests the growth or action of micro-organisms and is mostly used for preparations applied to living tissue (Rutala *et al.* 1997). Chlorination is usually employed as a low-level disinfectant to reduce the microbial burden in the water (Rutala *et al.* 1997).

The phenomenon that some pathogenic organisms survive water treatment with chlorination at conventional doses and contact times and enter the potable water reticulation system in a viable state is widely referred to as chlorination resistance in engineering and medical literature. This is not the same concept as is used in antibiotic resistance and should not be confused with the processes leading to antimicrobial resistance.

In many cases, protection of raw water sources from contamination is problematical and such water need to be treated to prevent the spread of pathogenic micro-organisms. The first-line processes known as 'conditioning of water for disinfection' are mainly coagulation, sedimentation and filtration. These processes render water suitable for the final barrier process, namely disinfection. Chlorine is by far the most commonly used disinfectant and in developing countries the use of chlorine is often the only affordable means of disinfecting drinking water.

Several factors influence the efficiency of disinfection with chlorine. These include pH and turbidity of the water, concentration of the chlorine and contact time (World Health Organisation. Guidelines for Drinking Water Quality. Recommendations, 1996). Calculating the optimal contact time of water to be disinfected with chlorine in large water treatment works is a complex exercise. For small-scale users e.g. farming operations and small rural treatment works of local water sources (wells, rivers) there are guidelines issued by the World Health Organization (Galal-Gorchev, 1996), for optimal disinfection of water for drinking purposes. The contact time at a pH of less than 8 and a maximum turbidity of 1 NTU is given as 30 minutes (Galal-Gorchev, 1996; National Health and Medical Research Council of Australia and New Zealand, 1996). Contact time should only be increased with due cognisance of the potentially harmful effects of over-chlorination, (Bull *et al.* 1995), but human health and safety should never be compromised.

There is increasing concern about the ability of rural landowners and smaller local authorities to treat raw water obtained from rivers downstream from dense settlements. Should chlorine resistance be present to any significant degree in organisms occurring in free-flowing waters, this will imply that the ability to disinfect raw water from such polluted rivers is seriously impaired. The presence of chlorine resistant organisms in treated water is an indication that water treatment to inactivate pathogens may be ineffective.

The levels of chlorine resistance have not been determined in any of the river systems in the Eerste River catchment area. This is a sensitive topic from the standpoint of tourism, agriculture and contact of the local population with the river water. The health implications of the presence of such resistant organisms are considerable. Thus, the aim of this study was to establish the possible presence of organisms resistant to chlorine in the river below Kayamandi as indicators of environmental hazard. Organisms resistant to chlorine will also be an indication of whether small-scale treatment of the water downstream from the sources of pollution as encountered in the

Plankenbrug River can be carried out successfully. If there is doubt about the ability of small-scale users such as farmers and small local authorities to treat the water, this will be a further indication of the extensive environmental damage inflicted by the pollution in the Plankenbrug river due to sanitation failures in the dense settlement of Kayamandi.

METHODS

E. coli was chosen as indicator organism for the determination of chlorination resistance.

The river was sampled downstream from Kayamandi ("Below Kayamandi"). The samples were collected in autoclaved sample bottles according to the guidelines set out by the South African Bureau of Standards (South African Bureau of Standards, 1984) that incorporates the standard methods set out by the American Public Health Association, American Water Works Association and the Water Environment Federation (American Public Health Association, American Water Works Association & Water Environment Federation, 1992 & 1995; American Society for Microbiology, 1997). The samples for this substudy were collected exactly as on all other sampling occasions and no sodium thiosulphanate was present in the sample bottles.

The standard methods to assess the resistance of organisms isolated from river water to chlorination disinfection were followed (Maurer, 1978; Harley & Prescott, 1996). The samples were mixed thoroughly, filtered and divided in 5 equal samples of 225 ml each into clean sterile bottles. The rest of the water in the original sample bottle was used as a control sample with no chlorine added. This control sample was tested for faecal coliforms and *E. coli* according to the multiple tube method as described previously. All determinations in this study were done in duplicate.

Freshly manufactured Medisure® chlorine powder (Medichem, Tokai, South Africa) was used. The powder was weighed to four decimals of a gram and added to the sample water in the bottles so that concentrations formed a range of total chlorine: 0.1 mg/l; 0.3 mg/l; 0.4 mg/l, 0.5 mg/l and 0.6 mg/l. This range of concentrations was selected after consultation with the Chief Superintendent of the Cape Town water treatment works. The concentration of available chlorine was verified by testing with a HACH 2000 spectrophotometer.

The bottles with water and chlorine mixture were left standing for 30 minutes. This is the minimum contact time for chlorination of small water sources (such as simulated in the present laboratory investigation) recommended by the World Health Organization (World Health Organisation. Guidelines for Drinking Water Quality. Recommendations, 1996) and adopted by many other countries (Galal-Gorchev, 1996; National Health and Medical Research Council and Agriculture and Resource Management Council for Australia and New Zealand, 1996).

After 30 minutes, samples from each of the bottles of water and chlorine mixture were subjected to standard test for faecal coliforms and *E. coli* as previously described and the samples were incubated overnight at 37°C.

One (1.0) ml of the contents of each of the bottles of sample water from the river plus chlorine was added to 9.0 ml non-ionic ethoxylated sorbitan ester (Tween 80, Merck Schuchardt). These bottles were left covered on the laboratory bench for 30 minutes. The bottles were agitated from time to time during the 30 minutes.

One (1.0) ml of the contents of each bottle containing water and chlorine mixture were added to 9.0 ml brain heart infusion broth (Biolab Merck). These tubes were incubated overnight at 37°C.

River water and chlorine mixture from each bottle was also plated out on MacConkey agar plates (Biolab Merck), blood agar plates (tryptose blood agar, Biolab Merck) and brain heart infusion broth. The MacConkey agar plates and brain heart infusion broth tubes were incubated aerobically overnight at 37°C. The blood agar plates were incubated in a 5% CO_2 incubator overnight at 37°C.

From the river water and chlorine mixture 1.0 ml) to which was added Tween 80 (9.0 ml) and left standing on the bench for 30 minutes, MacConkey agar plates, blood agar plates and brain heart infusion broth were inoculated. The MacConkey agar plates and brain heart infusion broth tubes were incubated overnight at 37°C. The blood agar plates were incubated in an incubator containing 5% CO_2 gas at 37°C.

The bottles of river water and chlorine were left standing a further hour on the bench (total exposure 90 minutes). These bottles were vortexed initially and then manually agitated from time to time.

After the extra hour, a sample from each bottle containing river water and chlorine mixture was subjected to the standard determinations for faecal coliforms and *E. coli*. A sample from each bottle was also plated out again on MacConkey agar plates and brain heart infusion broth which were incubated aerobically overnight at 37°C and on blood agar plates which were incubated in an incubator containing 5% CO_2 gas at 37°C.

RESULTS

After 30 minutes contact with chlorine

Table 5.4.1 *E. coli* organisms from water sampled "Below Kayamandi" on 11 June 2002 surviving after treatment with various chlorine concentrations for 30 minutes

Total chlorine concentration	**Faecal coliforms per 100 ml surviving**	***E. coli per* 100 ml surviving**
0 (control)	27 810	27 810
0.1 mg/*l*	4 930	4 930
0.3 mg/*l*	792	792
0.4 mg/*l*	3 290	3 290
0.5 mg/*l*	0	0
0.6 mg/*l*	2	2

After 90 minutes contact with chlorine

Results obtained from organisms isolated from water sampled "Below Kayamandi" on 11 June 2002 after treatment with various chlorine concentrations as used in the previous experiment, but after a further 60 minutes (90 minutes in total) are depicted below.

Table 5.4.2 *E. coli* organisms surviving various chlorine concentrations after 90 minutes

Total chlorine concentration	**Faecal coliforms per 100 ml surviving**	***E. coli* per 100 ml surviving**
0.1 mg/*l*	24	24
0.3 mg/*l*	12	7
0.4 mg/*l*	6	6
0.5 mg/*l*	0	0
0.6 mg/*l*	0	0

DISCUSSION

An anomaly occurred in the results of the number of organisms surviving after 30 minutes of contact with the chlorine. At 0.3 mg/l chlorine, 792 *E. coli* organisms survived, while at 0.4 mg/l, 3 290 organisms survived - an increase of 315% over the previous concentration rather than the expected reduction. These determinations were done in duplicate. It is not known why more organisms survived at the higher concentration. It may have simply been a factor of coincidental sampling selection of more resistant organisms.

It is clear from the results that after 30 minutes' contact time with the chlorine, *E. coli* organisms survived up to the highest concentration used in this experiment. Even after 90 minutes of contact time, organisms still survived up to 4 mg/l total chlorine. If a disinfection system utilising 30 minutes' contact time is used to disinfect such water, then chlorine resistance will develop. The fact that organisms still survived up to the level just below optimal after 90 minutes of contact time, strengthens this conclusion. Water from the Plankenbrug River is therefore not suitable for disinfection with the ordinary methods available to small-scale users without increasing either the chlorine concentration and/or the contact time. This has implications for human health as well as

for agriculture since irrigation water with a high chlorine concentration is phytotoxic to plants and affects animal health.

Local authorities aim to chlorinate bulk water supplies to a level of 0.5 mg/l water, but this concentration is very difficult to maintain over the entire length of the distribution line (personal communication: Chief Superintendent of the Cape Town water treatment works). If one keeps in mind that only 225 ml of river water was exposed to each concentration of chlorine, then the number of organisms that would have survived conventional chlorination of bulk water supplies sourced from the river would have been considerable.

Payment (Payment, 1999), evaluated transient failures or reductions in treatment efficacy in two water distribution systems in Canada. The experiments evaluated the survival, inter alia, of *E. coli* up to 0.7 mg/*l* total chlorine. His results suggested that the maintenance of a free residual concentration of chlorine in a distribution system did not provide a significant inactivation of pathogens, especially those represented by intrusions into the distribution system, or regrowth of organisms in biofilms along the distribution system. Cross-connections and back-flows can also introduce pathogens into the distribution system. Typically, those failures would result in infecting clusters of individuals, without eliciting the usual signs accompanying large outbreaks and thus remain undetected (Payment, 1999). These findings are of particular relevance for local authorities and other water users needing to use water downstream from dense settlements such as the one in the present study.

Antiseptics and disinfectants are extensively used in e.g. health care settings for the disinfection of a variety of topical and hard-surface applications (Bloomfield, 2002). Although there is a wide variety of active ingredients found in these products, chlorine figures prominently amongst them. The widespread use of such agents have prompted discussion on the development of microbial resistance, in particular whether antibiotic resistance is induced by disinfectants (Bloomfield, 2002).

Different types of micro-organisms differ in their response to antiseptics and disinfectants (Bloomfield, 2002; McDonnell & Russell, 1999; Meckes, 1982; Morozzi, Cenci, Caldini, Sportolari & Bahojbi, 1982; Russell, 2002). This is hardly surprising in the light of their different cellular structure, composition and physiology. There has been some recent work done on extending the classification of resistance of micro-organisms (Russell, 2002). The classification is now given as the following sequence (Russell, 2002):

Descending order of resistance to antiseptics and disinfectants (Russell, 2002)
(examples in brackets):

1: Prions*
(Creutzfeldt-Jacob disease, bovine spongiform encephalopathy)
↓
2: Coccidia
(*Cryptosporidium*)
↓
3: Spores
(*Bacillus*, *C. difficile*)
↓
4: Mycobacteria
(*M. tuberculosis, M. avium*)
↓
5: Cysts
(*Giardia*)
↓
6: Small non-enveloped viruses
(polio virus)
↓
7: Trophozoites
(*Acanthamoeba*)
↓
8: Gram-negative bacteria (non-sporulating)
(*Pseudomonas*, *Providencia*)
↓
9: Fungi
(*Candida, Aspergillus*)
↓
10: Large non-enveloped viruses
(Enteroviruses, Adenoviruses)
↓
11: Gram-positive bacteria
(*S. aureus, Enterococcus*)
↓
12: Lipid enveloped viruses
(HIV, HBV)

[* NOTE: The conclusions on prions are not yet universally agreed upon.]

The first three entries at the top of the resistance flow chart were not investigated in the present study, nor were numbers 5, 7 and 9. These organisms should receive research attention. In the case of Coccidia such as Cryptosporidium, efforts to do research on these organisms are seriously hampered by the technical difficulties in identifying them from water samples and culturing them *in vitro*. Organisms from genera listed under numbers 3, 4, 8, and 11 were isolated from water from the Plankenbrug River and their antibiotic resistance should also receive research attention.

In the light of the increased chlorine resistance in *E. coli* noted in the present study and the increased antibiotic resistance to some antibiotics noted in the organisms surviving the chlorination

treatment (see Table 5.5.2), the present preliminary study found support for concerns regarding induced antibiotic resistance in disinfection-resistant organisms.

Micro-organisms can adapt to a variety of physical and chemical conditions in the environment and reports of resistance (Bloomfield, 2002; McDonnell *et al.* 1999; Urassa, Lyamuya & Mhalu 1997; Meckes, 1982; Morozzi *et al.* 1982) to extensively used antiseptics and disinfectants could have been predicted on theoretical grounds. The most significant survival mechanisms are clearly intrinsic, in particular the ability to sporulate, adaptation of pseudomonads, and the protective ability of biofilms (Bloomfield, 2002). In these cases the term resistance may be incorrect and "tolerance" may be more correct. Tolerance is defined as developmental or protective effects that permit micro-organisms to survive in the presence of an active agent (Bloomfield, 2002). Reports of resistance (Bloomfield, 2002; Russell, 2002; Le Dantec, Duguet, Dumontier & Dubrou, 2002), have often paralleled issues such as inadequate cleaning, incorrect product use, or ineffective infection control practices and the contribution of these problems cannot be under-estimated. Chlorination, while initially lowering the total number of bacteria in sewage, may substantially increase the proportions of antibiotic-resistant potentially pathogenic organisms (Murray, Tobin, Junkins & Kushner, 1984). Furthermore, selective factors operating in the aquatic environment of a water treatment facility can act to increase the proportion of antibiotic-resistant members of the standard plate count bacterial population in treated drinking water (Armstrong, Calomiris & Seidler, 1982). The finding of possible chlorine resistant *E. coli* organisms in the present investigation is therefore a serious wake-up call. The present investigation was only a pilot study and large-scale investigations on more organisms should be instituted as a matter of urgency.

There are high requirements for asepsis and disinfection of public facilities (especially clinical settings such as hospitals), as well as for the disinfection of water and sewage. These matters should receive much more attention, especially if improper disinfection can also result in survival of antibiotic-resistant organisms. This situation causes a double jeopardy - the ability to eliminate these organisms from clinical settings are impaired as well as the ability to treat those persons infected by them. It is all the more worrying if the results of improper sanitation and disinfection are detected in free-flowing water, indicating that in restricted areas such as clinics, hospitals and water and sewage treatment works these organisms may be much more prevalent.

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GENETIC DIVERSITY OF *EHRLICHIA RUMINANTIUM* IN SOUTH AFRICA

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SUMMARY

Heartwater is caused by the tick borne pathogen *Ehrlichia ruminantium*. It is a disease that occurs in cattle, sheep and goats and in wild ruminants. The currently available blood vaccine is expensive to produce, requires an uninterrupted cold chain for delivery and provides only limited protection against heartwater. The vaccine is therefore not suitable for use in rural areas. Current investigations, by researchers at ARC-OVI, in vaccine development have indicated that considerable genetic diversity exists among isolates in the field. This explains the limited protection obtained with the current blood vaccine. These investigations have highlighted the importance of detailed knowledge of isolates present in the field for vaccine development. However, scant epidemiological information is available from areas particularly badly affected by the presence of heartwater in both the commercial and communal farming sectors. Therefore an in-depth epidemiological study is currently ongoing in heartwater endemic regions of South Africa. The specific and sensitive pCS20 diagnostic test was used to detect *E. ruminantium* in ticks and blood of domestic ruminants and sequencing of the corresponding pCS20 DNA fragment was used to distinguish between and to identify new isolates. Only 24 % of samples collected from heartwater endemic areas in South Africa tested positive for *E. ruminantium* and seven new isolates were identified. Two of these isolates were established in culture and a cross protection study indicated that they do not protect against the highly virulent Welgevonden isolate but that the Welgevonden isolate protects against them.

INTRODUCTION

Heartwater is an economically important disease for the red meat and wool industries in SA and it is caused by the tick borne pathogen *Ehrlichia ruminantium* (occurs in cattle, sheep and goats and in wild ruminants). The disease is widespread in Sub-Saharan Africa and Madagascar and it is also present on 3 neighboring islands in the Caribbean (Uilienberg G. 1996). Heartwater is transmitted by ticks of the genus *Amblyomma* of which *A. hebraeum* is the only known vector in South Africa (Bezuidenhout *et al*. 1987).

Currently, immunisation of animals relies on the infection and treatment method where animals are infected with blood containing the Ball3 isolate and treated with tetracycline once a febrile response is detected. This vaccine is expensive to produce, requires an uninterrupted cold chain for delivery and is therefore not suitable for use in rural areas. Because it contains live *E. ruminantium* organisms, it cannot be used outside S. African endemic areas. Furthermore, there is considerable genetic diversity among isolates in the field, which may explain the limited protection against heartwater obtained with the vaccine. Because of this many farmers practice dipping to reduce tick infestation.

The specific and sensitive pCS20 diagnostic test is used to detect *E. ruminantium* in ticks and blood of domestic ruminants (Van Heerden *et al*, in Press). Sequencing of the corresponding pCS20 DNA fragment is used to distinguish between and to identify new isolates. There is also a recombinant ELISA (the MAP1B) which detects animals which have had recent exposure to *E. ruminantium* but

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neither test will detect otherwise healthy carrier animals with 100 % certainty (Peter, T.F., and Jongejan, F. 1998).

Since only scant epidemiological information is available from areas particularly To establish new E. ruminantium cell-lines, the BA 886 (Yunker *et al*, 1988) cell line is used as host cells for *E. ruminantium*, and is propagated in DF-12 medium. Cells are cultured as monolayers at 37 °C in a humidified atmosphere of 5 CO_2 in air.badly affected by the presence of heartwater in both the commercial and communal farming sectors an in-depth epidemiological study is being undertaken in Heartwater endemic regions of South Africa. In this study 24 % of samples tested positive for *E. ruminantium* and seven new isolates were identified (by sequencing). Two of these isolates were established in culture. To establish new E. ruminantium isolate in culture the BA 886 (Yunker *et al*, 1988) cell line is used as host cells for *E. ruminantium*, and is propagated in DF-12 medium. A cross protection study indicated that only one new isolate protects against the highly virulent Welgevonden isolate but that the Welgevonden isolate protects against both. A geological information system was used to plot the new isolates on a map.

MATERIALS AND METHODS

Collection of samples

Amblyomma hebraeum ticks and blood samples from cattle, sheep and goats were collected from designated farms in heartwater-endemic regions of South Africa. These regions include the Eastern Cape, Kwazulu Natal, Mpumalanga, Limpopo, Gauteng and North West Provinces. Stabilates were prepared at a ratio of 1:1 using sucrose potassium glutamate (SPG) and blood. Live ticks were homogenised in 1.35 µl SPG.

Identification and Characterization of *E. ruminantium* in samples

The pCS20 PCR diagnostic test (specific for *E. ruminantium*) was used to determine the presence of *E. ruminantium* in the blood and ticks. PCRs were performed with 5 µl of either blood or tick sample as template in a 50 µl reaction with the primers HH1 (5'-CCC TAT GAT ACA GAA GGT AAC CTC GC-3') and HH2 (5'-GAT AAG GAG ATA ACG TTT GTT TGG-3'). The amplicon is ~900-bp within the 1,306-bp pCS20 sequence of *E. ruminantium*. The pCS20 DNA fragment of samples that tested positive with the pCS20 PCR was sequenced (ABI Prism 3100 Genetic Analizer Applied Biosystems. The TaKaRa EX Taq (TAKARA SHUZO CO., LTD Japan) enzyme was used. PCR conditions were: Incubation of 25 seconds at 94 °C, 30 cycles of denaturation at 94 °C for 30 sec, annealing at 62 ° for 45 sec, elongation at 72 °C for 30 sec, final elongation at 72 °C and hold at 4 °C (Gene Amp PCR System 9700, Applied Biosystems). Each set of PCRs included a positive control of 1 µl purified genomic DNA *of E. ruminantium* (Welgevonden) and a negative control containing 5 µl distilled H_2O (Van Heerden *et al*, in Press). The pCS20 positive samples were further characterised by PCR amplification and sequencing of the 16S gene.)

Establish new genotypes in culture

Infection of cell BA 886 monolayers

A merino sheep was infected with *E. ruminantium* by intravenous injection of 10 ml blood stabilate from a sick sheep from Springbokfontein. The Body temperature of the sheep was monitored daily and a blood sample was drawn, from the jugular vein into sterile Vac-u-test^R tubes containing heparin (lithium heparin, 14.3 USP/ml blood) as anticoagulant on the third day of febrile reaction when it rose to 42° C. The infective blood (3 ml) was inoculated into each of several 25 cm² culture flasks containing a confluent layer of BA 886 cells. The cultures were incubated at 37° C for 2 to 3 h

on a rocking platform at 3 cycles/min after which the infective inoculum was decanted and the cell monolayer was rinsed with 3 x 5 ml of PBS. Five ml DF-12 medium was added and incubation was continued.

Heavily infected cultures were harvested for sub cultivation by scraping off the cell monolayer into the medium using cell scrapers. Cells were dispersed by pipetting the suspension up and down with a variable pipette (0.5 to 5 ml, Socorex, Lausanne, Switzerland) and the suspension was then transferred at a ratio of 1:2 or higher on to new endothelial cell monolayers. After 24 h the entire medium was discarded and replaced with 5 ml fresh DF-12 medium.

Microscopic examinations were carried out after staining as follows in order to determine the presence of *E. ruminantium* in the endothelial cells. Small samples were removed from the monolayer using a sterile 21 gauge needle with a bent tip and smears were prepared which were air-dried, methanol-fixed and stained with RapiDiff (Clinical Sciences Diagnostics, Booysens, South Africa).

Determine cross-protection status of new isolates relative to other isolates

The new cultured isolates were titred in Heartwater free sheep. Cross-protection studies were done using the new genotypes, and the Welgevonden isolate (currently shows widest protection range). The immunised sheep received a heterologous challenge two months later and were monitored for signs of disease and a reaction index was determined (Collins NE *et al.* 2003). The sheep that were used for this study were monitored for temperature reaction every day and sick animals were treated with tetracycline.

RESULTS

A total of 574 samples of blood and ticks were probed with pCS20. Of these only 47 blood samples and 92 tick samples probed positive with the pCS20 probe. Nineteen of the pCS20 positive samples were further characterised by sequencing the corresponding pCS20 fragment. Seven new isolates were identified and their locatia indicated in fig.1. The pCS20 sequence of five samples from different farms was identical to that of the Rockhurst isolate. (Table 1) and 1 of each of the following isolates: Silverdale, Argyll, Glendowan1, Glendowan2, Springbokfontein1 (SBF) and Springbokfontein2. Eight samples were also characterised by sequencing the 16S gene (Table 1). Interestingly, 2 of the samples with Rockhurst pCS20 sequence had different 16S genes (Table 1). Comparison of the Rockhurst isolate with the Blaauwkrans, Mara87/7 and Welgevonden isolates show diversity in the sequence (from basepare (bp) 685). The new isolate and the existing Blaauwkrans isolate sequence was identical from the start of the sequence to base pair 684 and from base pair 685 to the new Rockhurst isolate and Mara87/7 was identical (Table 2). This suggests that Rockhurst could have resulted from a recombination of Blaauwkrans and Mara87/7 isolates (Table 2).

The two SBF isolates were successfully cultured and tested in a cross protection study. Neither of these is more virulent than the Welgevonden isolate although SBF2 does cross-protect against Welgevonden challenge.

Table 1. Characterisation of isolates occurring in ticks and blood collected from farms in heartwater endemic areas in South Africa.

Province	Town	Farm	Sample	pCS20	16S
				Sequence#	Sequence#
MP	Eglington	Dip tank	Tick	Mara87/7#	ND

EC	Grahamstown	Rockhurst	Blood	Rockhurst*	Welgevonden
EC	Grahamstown	Henley	Tics	Rockhurs*t	ND
EC	Grahamstown	Riverside	Tick	Rockhurst*	ND
EC	Grahamstown	Riverside	Blood	ND	Omatjenne
EC	Grahamstown	Henley	Tick	Mara87/7#	ND
EC	Grahamstown	Glendowan	Tick	Glendowan1*	ND
EC	Grahamstown	Glendowan	Tick	Glendowan2*	ND
EC	Grahamstown	Glendowan	Tick	Mara87/7#	ND
EC	East London	Little Chenunga	Blood	Rockhurst*	Mara87/7
EC	East London	Sunny Side	Blood	ND	ND
EC	East London	Fountain Spr	Blood	Mara87/7#	ND
EC	East London	Silverdale*	Tick	Silverdale*	ND
EC	East London	Blow Ridge	Tick	Blaauwkrans#	ND
EC	East London	Blow Ridge	Tick	Rockhurst*	ND
KZN	Msinga	Dip tank	Blood	ND	Omatjenne
KZN	Amandlalathi	Dip tank	Tick	Mara87/7#	Mara87/7
KZN	Port Shepstone		Tick	Mara87/7#	Mara87/7
KZN	Vryheid	Argyll	Blood	Argyll*	ND
GAUT	Pretoria	Roodeplaat	Blood	Kumm2#	ND
LIMP	Potgietersrus	SBF	Blood	SBF1*	Welgevonden
LIMP	Potgierersrus	SBF	Blood	SBF2*	Welgevonden

* - First isolated on this farm ND – not done # - sequence identical to stock X
SBF –Springbokfontein

Table 2. Identification of the diversity between the New Rockhurst isolate with exiting isolates

	bp 191	bp 210	bp 243	bp 320	bp 380	bp 616	bp 685	bp 694	bp 704
Blaauwkrans	G	A	C	C	G	T	T	C	G
Rockhurst	G	A	C	C	G	T	C	T	A
Mara87/7	G	A	C	T	G	C	C	T	A
Welgevonden	A	C	T	T	A	C	T	C	G
	B	B	B	B	B	B	M	M	M

bp – base pair B – Blaauwkrans isolate M – Mara87/7 isolate

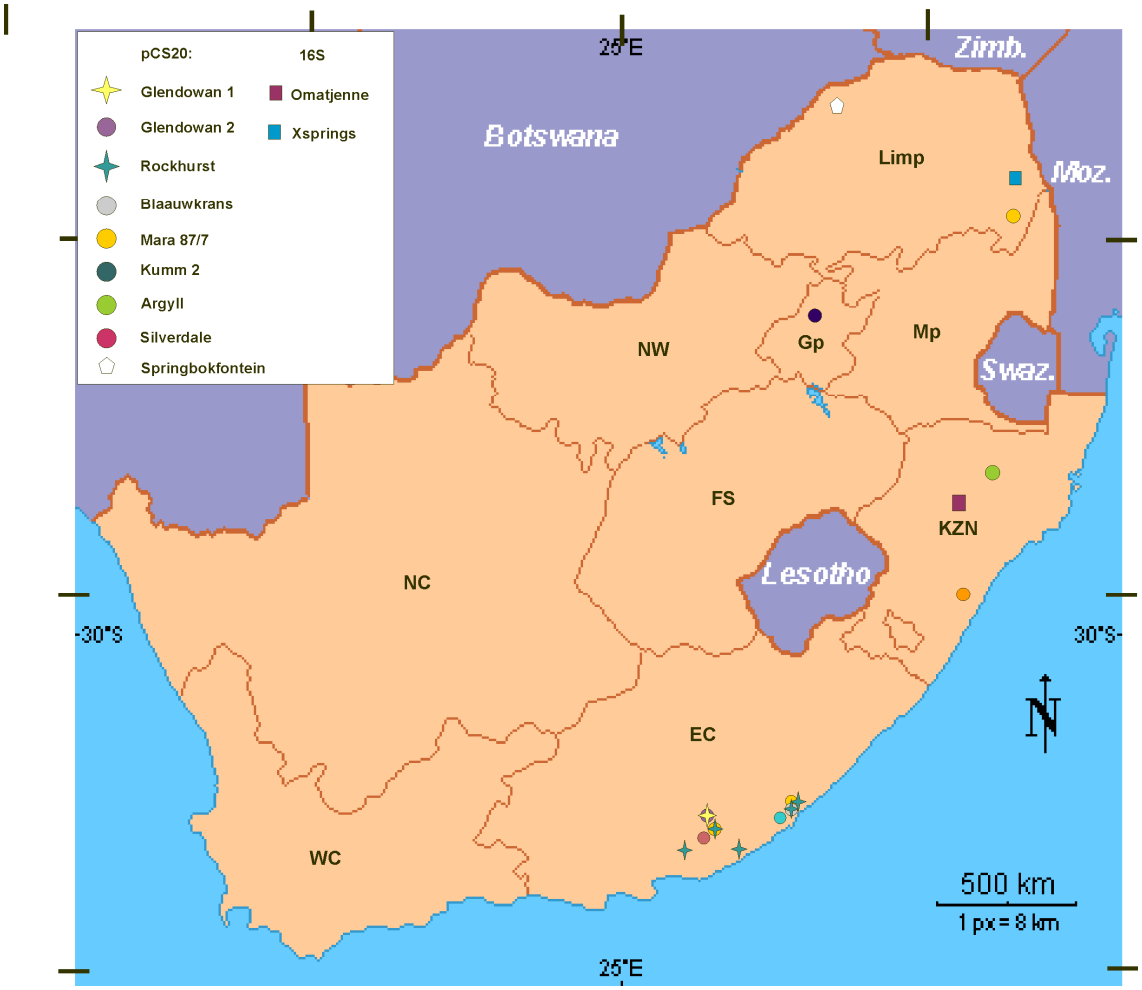

Figure 1. Geological distribution of Heartwater

In conclusion, of the 19 samples of blood and ticks, which were further characterised by pCS20 sequencing, five were new isolates. These isolates were named after the farm where they were first isolated. The new Rockhurst isolate was identified on five different farms. The following isolates was discovered: Silverdale, Argyll, Glendowan1, Glendowan2, Springbokfontein1 (SBF1) and Springbokfontein2 (SBF2).

The 16S gene was sequenced from 9 samples. These samples were identical to either the Welgevonden, Mara87/7 or Omatjenne isolates.

DISCUSSION

The identification of seven new isolates the Rockhurst, Silverdale, Argyll, Glendowan1, Glendowan2, Springbokfontein1 (SBF1) and Springbokfontein2 (SBF2) shows that genetic diversity of *E. ruminantium* isolates do exist in the field.

The cross-protection study undertaken between the well characterised Welgevonden stock and the two new SBF1 & SBF2 isolates indicated that only the SBF2 isolate cross protected. SBF2 is the first isolate that has been found to protect animals against a challenge with the Welgevonden stock. Unfortunately a similar study was not undertaken with the vaccine Ball3 stock. However, we do know that animals that are immune to the Ball3 stock (vaccine stock) succumb to heartwater when challenged with the Welgevonden stock (Collins NE *et al.* 2003). Therefore it will be highly likely

that animals immune to Ball3 stock would similarly succumb to heartwater when challenged with the SBF2 isolate.
The data presented here highlight the genetic diversity of isolates and explains why only limited protection is obtained with the blood Ball3 vaccine in the field. Continual characterisation of new isolates will benefit the development of an efficient vaccine against heartwater.

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SERUM ZINC LEVELS OF SHEEP AFFECTED BY ULCERATIVE BALANOPOSTHITIS AND VULVOVAGINITIS

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SUMMARY

Ulcerative balanoposthitis and vulvovaginitis of sheep is a major problem in the extensive grazing areas of the Northern Cape, Free State and Western Cape provinces of South Africa. It is mainly a venereal disease affecting the Dorper and other non woolled sheep to a greater extend. It is caused by an infection with strong evidence that Mycoplasma mycoides mycoides (Large Colony) is a primary agent in the etiology.

The disease causes inflammation, erosions and ulceration of the mucous membranes of the external genitalia of rams and ewes. The nature of the lesions indicates that the integrity of mucous membranes could possibly be compromised and zinc deficiency might be incriminated.

Serum levels of zinc were determined in 17 infected and 7 healthy flocks and the levels of zinc from affected sheep (n=147) were compared with levels from healthy sheep (n=422). The mean serum zinc value of all the sheep was 0.946µg/ml,
(s= 0.274) slightly below the middle of the normal range of 0.7 to 1.3 accepted by South African laboratories.

The frequency distribution of the zinc values was not normal but non parametric methods did detect that the median of zinc levels in the affected sheep were significantly higher than the levels in the unaffected sheep ($P = 0.004$).

The sample of means from affected farms also showed significantly higher serum zinc level than the sample of means from farms with no clinical case of UBP.
(P=0.014)

The median values of zinc serum levels from sheep on farms affected by UBP were significantly higher than the median values from farms with no clinical cases.
(P=0.018)

The degree of lesions scored on an ordinal scale of 0 to 6, based on degree of inflammation, secondary infection, and extent of mucosal damage did not correlate significantly with the serum zinc levels, neither on cumulative samples nor on individual farm samples.

INTRODUCTION

Ulcerative balanoposthitis and vulvovaginitis of sheep is a major problem in the extensive grazing areas of the Free State, Northern-, Eastern- and Western Cape provinces of South Africa. It is mainly a venereal disease affecting the Dorper and other non woolled sheep to a greater extend. It is

caused by an infection with strong evidence that Mycoplasma mycoides mycoides (Large Colony) is a primary agent in the etiology.

The disease causes inflammation, erosions and ulceration of the mucous membranes of the external genitalia of rams and ewes. The nature of the lesions indicates that the integrity of mucous membranes could possibly be compromised and zinc deficiency might be incriminated.

Trichard et al 1993 described the clinical manifestation of the disease as follows:
Lesions developed 4 – 10 days after the introduction of rams to the ewes for breeding. Reluctance or complete refusal to serve, is the first sign of the disease in rams. Some animals are depressed and may stand to one side with their backs arched. If still inclined to mate, free blood discharges from the preputial opening. The skin around the prepuce is often blood –stained and haemorrhaging may occur after urinating. The prepuce is swollen, erosions occur at the mucocutaneous junction and on extrusion the penis is hot, painful to the touch and the mucous membrane may easily tear and bleed. Small, scattered erosions are visible. At times the erosions are extensive, often forming ulcers covering most of the glans and the processus urethralis. Paraphimosis may occasionally develop. This is followed by extensive trauma and soiling of the penis. In primary outbreaks up to 100% of the rams may be affected.
Some ewes presented blood – stained hindquarters, particularly around the vulva and tail area. In Dorper sheep where the tail is docked short and the vulva is visible, the vulvar lips are swollen, oedematous and red, while blood – stained fluid may ooze from the external orifice. The vaginal mucous membrane bleeds easily when handled, particularly after vaginoscopic examination. Frequent wriggling of the tail-butt and urination is seen in some cases. Flies are attracted to the inflamed genitalia which further agitates the animals. After the acute signs have abated, scab covered ulcers are visible on the vulvar lips particularly ate the mucocutaneous junction and the ventral commissure. In the primary outbreaks the morbidity may reach 70 – 80 %."

MATERIALS AND METHODS

Study population

Serum from 569 Dorper sheep in the Northern Cape province of South Africa were tested, the majority originated from flocks in the Namaqualand district. Flocks of farmers that complained to the Springbok state veterinary office about UBPVV problems, during June 2001 to December 2002 were investigated. Clinical cases and comparable control cases were selected. During routine fertility examinations, a represent able sample was taken from flocks with no clinical cases of UBPVV.

Case definition

A flock of sheep where one or more of the rams is clinically infected with Ulcerative balanoposthitis, is considered as an infected farm. A ram is considered as clinically affected when on extrusion and close examination of the penis, pustules, vesicles, erosions or a red, swollen or bleeding penis is found.
Non-cases would be all flocks of sheep where none of the rams on examination of the extruded penis show any of the described symptoms.
No laboratory tests are available to confirm infection and clinical evaluation of lesions are the only method of diagnoses.

Sampling methods

5 to 7 ml of blood was collected from the jugular vein into vacuum glass bottles marked with the number or description of the sheep. Rubber stoppers were screened off with commercial plastic material before sealing the bottles. After complete coagulation, the serum was transferred to another marked glass tube with a rubber stopper screened off. The serum was send to the laboratory by courier in cooled containers.

Observations

Penis and vulva lesions were recorded. 45 cases were scored on a scale of 0 to 6 with reference to the degree of inflammation, mucosal damage (erosions, ulcerations), secondary infection, chronic lesions and the presence of phimosis or paraphimosis.

Laboratory analysis
The samples were analyzed by the Stellenbosch Veterinary Laboratory according to a modified version of the method described by Weisman in 1974.The laboratory was not informed about the disease status of the flocks.
Summary of method:
Clotted blood samples were centrifuged at 3000 rpm for 10 minutes and the serum removed within six hours of collection and stored at –20 °C until analysis. Five millilitres of 7 % Trichloroacetic acid (TCA) was added to 1ml of serum and allowed to stand for 10 minutes. After centrifugation at 4000 rpm for 15 minutes, the supernatant was decanted and centrifuged a second time at 3000 rpm for 10 minutes to pellet any remaining protein precipitate. Copper and zinc standards were prepared in the same manner as the samples. The concentration of copper and zinc was determined by atomic absorption spectrometry.

Data analysis
Microsoft Excel, NCSS and Arcview software were used to analyze the data.

RESULTS

Serum levels of zinc were determined in 17 infected and 7 healthy flocks and the levels of zinc from affected sheep (n=147) were compared with levels from healthy sheep (n=422). The mean serum zinc value of all the sheep was 0.946µg/ml,
(s= 0.274) slightly below the middle of the normal range of 0.7 to 1.3 accepted by South African laboratories.

Descriptive Statistics			Standard	
Variable	Count	Mean	Deviation	Median
UBP=0	427	0.9236161	0.2797642	0.92
UBP=1	147	1.010816	0.274734	0.95

Evaluation of repeatability
30 duplicated samples rendered a mean difference of 0.12 µg /ml between the paired samples, with a variance of 0.01.The standard deviation of the 60 repeated results was 0.27. When this was taken as the limits of agreement, the percentage of agreement was 90%. A Kappa value of .71 was calculated for the repetition tests when the two sets of samples were regarded as two observers.

Frequency Distribution
The distribution of the data was not normal, probably because of the large number of samples and the clustered origin of the samples.

Comparison of serum zinc levels from sheep with clinical UBP and sheep with no lesions of UBP.
Non parametric methods had to be used and an analysis with the Mann-Whitney U or Wilcoxon Rank-Sum Test for Difference in Medians was performed.
It was found that the median of zinc levels in the affected sheep were significantly higher than the levels in the unaffected sheep ($P = 0.004$).

Comparison of serum zinc levels from infected farms with levels from farms with no clinical cases.
Descriptive Statistics Section

Standard

Variable	Count	Mean	Deviation
UBP=0	7	0.8128572	0.1759464
UBP=1	17	1.004118	0.1825534

An equal variance T test was performed.
The sample of means from affected farms showed significantly higher serum zinc levels above the sample of means from farms with no clinical cases of UBP.(P=0.014) At an alpha level of 0.05, Power was 0.74.

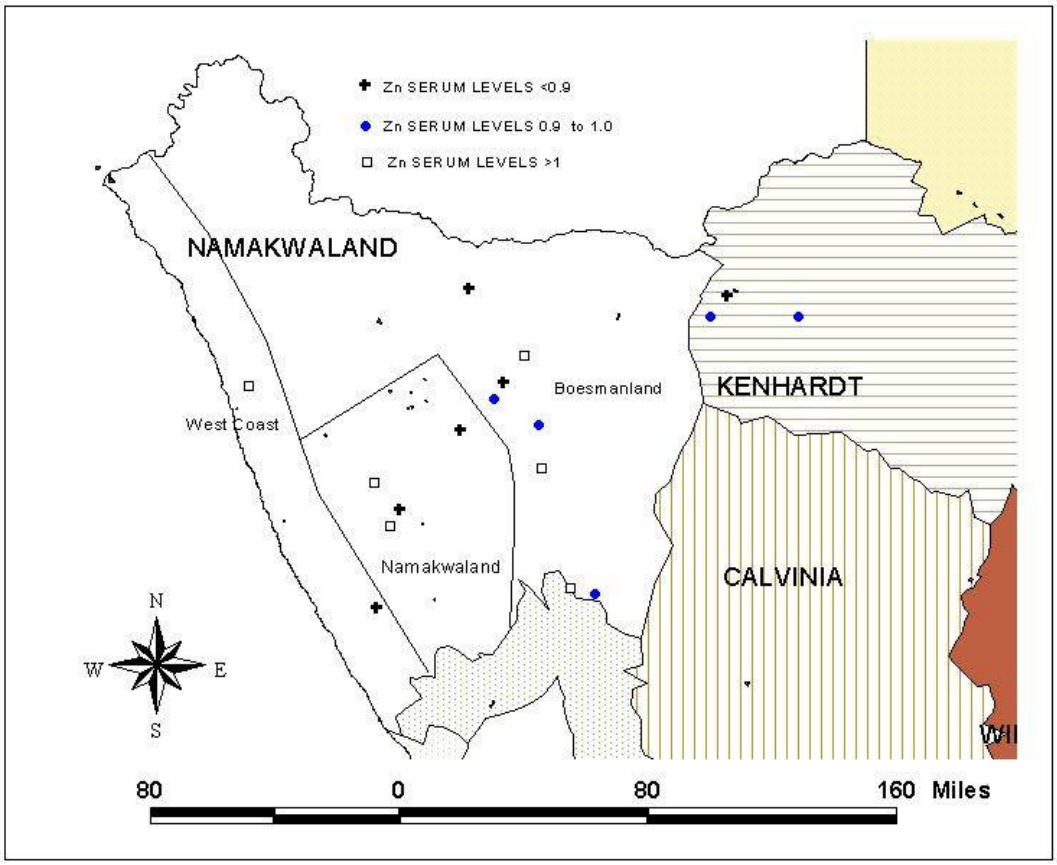
Median Statistics

Variable	Count	Median
UBP=0	7	0.83
UBP=1	17	0.98

Again a Mann-Whitney U or Wilcoxon Rank-Sum Test was done to analyze the difference in medians.
At α=0.05, the median values of zinc serum levels from sheep on farms affected by UBP were significantly higher than the median values from farms with no clinical cases. (P=0.018)

Correlation between severity of lesions and serum zinc levels
The degree of lesions scored on an ordinal scale of 0 to 6, based on the degree of inflammation, secondary infection, and extent of mucosal damage was subjected to a regression analysis against the serum zinc level, but the hypothesis that the slope was zero could not be rejected. Therefore it was concluded that the degree of the lesions did not correlate significantly with the serum zinc levels, neither on cumulative samples nor on individual farm samples.

Evaluation of values falling outside the normal range.
Values below 0.7 µg/ml and above 1.3µg/ml were examined in affected sheep, all sheep on infected farms and sheep on control farms. The proportion of sheep with a zinc level below 0.7 was significantly higher in sheep on control farms than on affected farms.(P<.01) Differences in proportions between the other groups were not significant.
Geographical considerations



The area from which the samples originate is an arid, mineral reach part of the Northern Cape Province of South Africa with extensive mining activity for inter alia zinc and copper. Three different components can be distinguished namely the west coast, mist belt with Karoo type shrubs, the winter rain Namakwaland area with a rich variety of shrubs and the dry, summer rain Boesmanland with extremely homogenous grassland. Despite the vast differences in the topography, soil composition, rainfall and vegetation, the serum zinc levels of sheep grazing the area did not reflect the differences. To a great extend the seasonal movement of sheep between the different areas would explain the homogeneity. If the higher and lower tails of the serum zinc levels are tabulated according to the locality where they were at the time of their examination, a remarkable even distribution is demonstrated.

Serum zinc level	Coastal farms	Namakwaland farms	Boesmanland farms
<0.9	1	2	3
>1.0	1	2	3

Nearest neighbor comparisons also demonstrate that high and low serum zinc levels were often obtained from sheep in the same area. 80% of mean measurements were closer to different category measurements than they were to measurements of the same category, indicating a random spread.

DISCUSSION

It is generally accepted that zinc stabilizes membranes. An antioxidant effect by protection of sulfhydryl groups in membranes is suggested. When plasma zinc decreases, it is believed that zinc dissociates from the membrane allowing association of cations leading to the oxidation to the disulfide form that does not bind zinc readily. The membrane protective effect of zinc may also involve the lipid component of the membrane. Hormone receptors and absorption sites could be affected. Intercellular binding is also reported to be dependant upon zinc. The inflammation, erosions, bleeding and ulceration seen in Ulcerative balanoposthitis and vulvovaginitis does create the impression that the mucous membranes are very sensitive.

The manifestation of UBPVV is often not explained by exposure to infection alone. A clear-cut demonstration of low zinc levels in sheep infected with UBPVV would have answered some of the discrepancies in the epidemiology of the disease. The finding that in fact zinc serum levels might be higher in affected sheep, raises the question that high zinc levels could lead to other mineral imbalances that could predispose to UBPVV.

The reliability of serum zinc determination compared with liver samples as an indication of deficiency had been considered, but it seems that both values are affected by physiological and pathological conditions. The serum samples were readily available during examination of sheep and could reflect the status at the time that UBP was evaluated.

It had been reported that serum (and liver) values of zinc does not correspond well with the clinical response obtained by supplementing zinc by the parental route. A number of farmers in the study also reported improvement and faster recovery from UBP after treatment with an injectable zinc preparation, but no controlled evaluation was done.

The skewed frequency distribution necessitated the use of non-parametric methods, and elimination of confounding factors was limited. Possible confounding factors could be different management of reproduction, feeding, mineral supplementation and many other factors on each farm. Soil and vegetation differences will have to be examined closer.

Working with the sample of means of the farms in the study improved the normality of the distribution but certain criteria were still not met. The smaller sample size on this evaluation reduced the significance of the findings.

The mere evaluation of a trend does not warrant a decrease in alpha and in fact the power could have been increased by using a higher alpha level.

Very low zinc levels were recorded on a number of control farms, but the risk of exposure to infection on these farms was unknown.

Zinc supplementation had been shown to decrease plasma copper levels in cattle. Zinc supplementation was also effective in treating copper poisoning in sheep. Therefore a negative effect of high levels of zinc in sheep, in a mineral rich part of the country, might be deleterious by inducing other mineral imbalances.

The careful monitoring of zinc levels after supplementation to evaluate the response would be valuable. Comparisons of zinc levels before and after UBP infections set in can also give an indication of the significance of zinc as a predisposing factor.

The tendency of serum zinc levels being higher on affected farms and in affected sheep was demonstrated by a number of analysis methods and can certainly not be ignored, although it should be interpreted with care.

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Appendix A. Data stratified by month of purchase and rate of liver fluke damage

Month	Liver damage	Negative	Grade1	Grade 2	Grade 3
March	Number	8	0	0	0
	In mass	168 (16)			
	Out mass	369 (36)			
	ADG	1.16 (0.24)			
	Days fed	175 (7)			
April	Number	400	4	9	7
	In mass	170 (28)	159 (8)	173 (23)	168 (13)
	Out mass	357 (33)	354 (24)	376 (16)	343 (33)
	ADG	1.24 (0.23)	1.23 (0.13)	1.31 (0.09)	1.21 (0.19)
	Days fed	152 (14)	159 (10)	155 (17)	145 (6)
May	Number	1762	1	72	97
	In mass	196 (34)	185 (-)	200 (33)	215 (38)
	Out mass	362 (34)	317 ((-)	368 (37)	367 (38)
	Days fed	128 (17)	127 (-)	132 (16)	120 (14)
June	Number	2088	6	68	64
	In mass	209 (41)	228 (48)	200 (34)	224 (31)
	Out mass	370 (39)	370 (57)	371 (35)	374 (45)
	ADG	1.47 (0.31)	1.26 (0.67)	1.45 (0.29)	1.56 (0.37)
	Days fed	111 (20)	110 (20)	119 (17)	97 (15)
July	Number	2276	13	136	31
	In mass	209 (52)	211 (32)	204 (39)	263 (80)
	Out mass	362 (45)	377 (35)	359 (38)	388 (56)
	ADG	1.56 (0.32)	1.71 (0.19)	1.53 (0.28)	1.55 (0.47)
	Days fed	99 (20)	98 (16)	103 (17)	83 (26)
August	Number	2136	5	101	10
	In mass	219 (51)	226 (25)	223 (47)	250 (65)
	Out mass	364 (44)	333 (36)	368 (43)	378 (42)
	ADG	1.67 (0.34)	1.39 (0.31)	1.69 (0.30)	1.70 (0.19)
	Days fed	88 (18)	73 (25)	87 (18)	76 (21)
September	Number	1482	6	139	10
	In mass	233 (47)	231 (45)	244 (61)	283 (60)
	Out mass	359 (41)	345 (26)	365 (41)	394 (37)
	ADG	1.68 (0.36)	1.82 (0.31)	1.63 (0.35)	1.63 (0.27)
	Days fed	76 (17)	65 (18)	74 (18)	68 (18)
October	Number	475	2	53	16
	In mass	265 (62)	358 (110)	290 (78)	278 (89)
	Out mass	370 (50)	430 (74)	383 (54)	377 (66)
	ADG	1.65 (0.42)	1.24 (0.16)	1.59 (0.41)	1.45 (0.46)
	Days fed	63 (14)	57 (21)	57 (14)	67 (16)
November	Number	2465	1	29	4
	In mass	340 (76)	410 (-)	377 (63)	373 (76)
	Out mass	399 (70)	409 (-)	419 (60)	429 (73)
	ADG	1.36 (0.58)	0.04 (-)	1.31 (0.47)	1.41 (0.56)
	Days fed	43 (12)	28 (-)	36 (10)	40 (4)
December	Number	47			
	In mass	400 (74)			
	Out mass	424 (86)			
	ADG	1.10 (0.56)			
	Days fed	28 (5)			

POSTERS

OVINE ENZOOTIC ABORTION: DISEASE CHARACTERISTICS AND ASPECTS VACCINATION CONTROL IN SOUTH AFRICA

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Ovine enzootic abortion (OEA) is caused by an obligate intracellular bacteria *Chlamydophila abortus* (formerly the ovine subtype of *Chlamydia psittaci*). *C. abortus* is widely distributed throughout the world causing abortion and foetal loss in sheep, goats and cattle. The majority of aborting ewes are young animals, although sheep of all lamb-bearing ages are potentially at risk of infection. *C. abortus* infection occurring for the first time in non-vaccinated pregnant ewes may cause 30% to 60% lamb mortality. It is common that about 5-10% of ewes abort annually in vaccinated flocks, as well as, where the infection is established as an enzootic disease. *C. abortus* infection resulting in abortion leads to effective immunity in affected ewes. Transmission to humans and spontaneous abortion in women caused by Chlamydia has also been documented. In South Africa, sporadic cases and outbreaks of OEA in sheep are reported in many sheep rearing areas of the country. The economic importance of the disease on the ruminant industry however, cannot be assessed due to the lack of epidemiological data.

Aspects on the signs and transmission of the infection, diagnosis, the immune response and vaccination control will be highlighted.

JOHNE'S DISEASE: GUDAIR VACCINE TRIAL

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ABSTRACT:

Johne's disease has a crippling economic effect on sheep farmers in the Overberg and Swartland regions in the Western Cape province. Some farmers report an annual cause specific mortality rate of up to 14%. In this region the disease progressed in such a manner that whereas in 1996 and 1997 predominantly older sheep (full mouth and older) were affected, currently even sheep younger than a year show typical symptoms of the disease.

The Directorate: Veterinary Services, Western Cape province, together with Virbac Animal Health, conducted a vaccine trial on 3 affected farms in the problem areas. The vaccine (Gudair®) is currently in use in Australia, New Zealand and Spain, where its efficacy in reducing mortalities has been proven. It is known that Gudair® vaccine causes lesions at the injection site in a significant number of animals. The purpose of this trail was to evaluate the severity of these lesions in herds with high infection rates and herds with a lower level of infection. In addition to measuring and evaluating the lesions, the weights of the sheep in both the sample and control groups were recorded.

150 Sheep on each of the three farms (50 adults, 50 six-months old, and 50 lambs of approximately 4 weeks of age) were vaccinated. The control group consisted of 150 additional sheep within the specified age groups. During the 300 days of the trial, significant lesions were recorded in 98% of animals with 14-26% showing abscesses or open wounds at 30 days post vaccination. The subsequent reduction in lesion size was variable and unpredictable. The average weights of the animals showed fluctuations according to the drought situation, but there were no significant differences between the sample and control groups.

COMPARISON OF SOLID PHASE COMPETITION ELISA AND LIQUID PHASE BLOCKING ELISA FOR DETECTION OF ANTIBODIES TO THE STRUCTURAL PROTEINS OF THE SAT TYPES OF FOOT-AND-MOUTH DISEASE VIRUS

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The internationally accepted tests for the detection of antibodies to the structural proteins of foot-and-mouth disease virus (FMDV), as prescribed by the Office International des Epizooties (OIE), are the virus neutralisation test (VNT) and the liquid-phase blocking ELISA (LpBE). While the VNT is concidered to be the "Gold Standard," the LpBE is more commonly used for the routine screening of large numbers of sera. However, studies have indicated that the LpBE can give false-positive results that may vary according to the animal population under consideration, and can be as high as 18% in stressed animals. Due to these problems, a solid-phase competition ELISA (SpCE) was developed by Mackay *et. al.* (2001), and compared with the LpBE and the VNT. The specificity of the SpCE was considerably higher than the LpBE and comparable to that of the VNT. Furthermore, the SpCE is easier to use and more robust and therefore offers an improvement for FMDV antibody detection. The test has now been proposed as the preferred ELISA by the OIE.

The test is currently not available in kit form, and has to be developed and validated for the SAT serotypes prevalent in sub-Saharan Africa. Test conditions have been optimized by cross titrations of the reagents used in assay. These include the antigen, the guinea-pig typing serum and the conjugate. The conditions for the test have further been investigated using a panel of known control sera. Initial experiments indicate that less variation occurred in the results of the SPcE with different concentrations of reagents, than was the case with the LpBE. This indicates the robustness of the SpCE.

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