

Genetic diversity of *Ehrlichia ruminantium* epidemiology in South Africa - an update

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Abstract

Heartwater is caused by the tick borne pathogen *Ehrlichia ruminantium*. It is a non-contagious disease that occurs in cattle, sheep, goats and wild ruminants. The disease is prevalent from the Eastern Cape to the Limpopo province in South Africa. Cattle, sheep and goats form a major contribution to rural and commercial livelihoods in South Africa and one of the major constraints to the survival of these animals is heartwater. Furthermore, heartwater is on the OIE's list of notifiable diseases because of its economic impact on rural livelihoods in Africa (<http://www.oie.int>). One of the problems affecting control of this disease was recently revealed by biotechnology as it has been found that there are several distinct genotypes in the field. Furthermore, the widely used live blood vaccine does not protect against several of these different genotypes. An extensive epidemiological study will aid in acquiring information on the characteristics of the different genotypes present in the field so that appropriate prevention and control strategies, based on these findings, can be implemented. The progress of participatory field studies, sampling of livestock blood and ticks (*Amblyomma hebraeum*), *E. ruminantium* genotype characterization and development of a new diagnostic tool for faster identification of heartwater infection in the field, are reported.

Introduction

Ehrlichia ruminantium is a rickettsial agent that is transmitted to livestock and wild ruminants by the tick species *Amblyomma*. It is the causative agent for the disease known as heartwater or cowdriosis (Cowdry, 1925a and b). Heartwater is prevalent in sub-Saharan Africa, including the African islands of Sao Tome, Zanzibar, Comores, Madagascar and Mauritius (Uilenberg, 1996). One of the problems affecting control of this disease was revealed by biotechnology as it was found that there are at least 15 distinct genotypes in the field in South Africa (Steyn, 2004). The prevention and control of heartwater necessitates knowledge of the characteristics of the different types of disease caused by the different genotypes, to discover epidemiological differences (host-agent-environment triad) and to implement appropriate prevention and control based on these findings. Genetic diversity of genotypes in the field may also affect the virulence of isolates. This has an influence on the cross-protection and immunity of ruminants in different heartwater endemic areas. Therefore, establishing the new isolates in culture will provide a valuable source for further characterization and cross-protection studies. New isolates that show the greatest range of cross-protection to other isolates will be suitable for future vaccine development studies.

Various techniques i.e. electron microscopy, fluorescence microscopy, and serology tests, pCS20 PCR and probing, 16S rRNA PCR and probing have been used to detect *E. ruminantium* in blood and tick samples from ruminants (Allsopp, *et al.*, 1998, Du Plessis, *et al.*, 1993, Van Heerden, *et al.*, 2004). The pCS20 PCR method has proved to be the most specific and sensitive of the diagnostic assays. PCR has also been the diagnostic tool for detection of DNA of various other parasites, in blood of sick or carrier animals and tick samples, and has gained wide acceptance because of its sensitivity and simplicity (Contini *et al.*, 2005). Although the pCS20 PCR assay and the probe is a sensitive test, it is labor intensive, difficult to quantify and has the potential risk for sample cross-contamination due to the requirement of numerous sample handling steps. For an epidemiology study thousands of samples need to be tested and the pCS20 PCR and probing is just too time

consuming. Therefore a real time PCR diagnostic test was designed that is faster and cheaper to use. Real-time quantitative PCR is the latest technique used for the detection of a variety of diseases (Contini *et al.*, 2005). The real-time PCR assay has the advantage over the PCR and probe assay in that it requires less manipulation, reduced amplification product carryover and no handling of radio active material.

The aim of this study was to use the pCS20 diagnostic real-time PCR (TaqMan) method for detecting the presence of *E. ruminantium* in blood and ticks (*A. hebraeum*), identifying new isolates of heartwater and investigating cross protection between isolates.

Materials and methods

Samples

Blood and *A. hebraeum* tick samples (n=2910) from cattle, sheep and goats were randomly collected from the endemic areas of South Africa. Blood was collected into sterile Vacutainer^R tubes containing EDTA as anticoagulant. Ticks were collected in plastic containers with holes in the lids and washed in 70 % Ethanol and were homogenized 1:1 (v/v) in sucrose potassium glutamate (SPG) and stored in Liquid Nitrogen until used.

Identification

DNA was extracted from ticks using the tissue culture protocol from QIAamp DNA kit (QIAamp) according to the manufacturer's instructions and DNA from the blood samples was extracted using the Generation capture column kit (Gentra systems) according to the manufacturer's instructions. A rapid and sensitive molecular diagnostic technique, the real-time PCR (TaqMan), was developed for the detection of *E. ruminantium* in field samples from ruminants and ticks. The sensitivity of the pCS20 PCR/probe was compared with that of real-time PCR (TaqMan). Sequencing of the corresponding pCS20 DNA fragment was used to distinguish between and to identify new isolates. Because the real time PCR (TaqMan) only amplifies a 200 bp amplicon the pCS20 PCR (900 bp PCR amplicon) was used to amplify the positive samples for sequencing. A nested pCS20 PCR was used to amplify low concentration samples for sequencing.

Results

Primers and a probe were designed using the pCS20 *E. ruminantium* gene region for the real-time PCR (TaqMan). The real time PCR amplifies a 200bp amplicon. This new assay was found to be more sensitive (100 fold) than the pCS20 PCR/probe when using Welgevonden genomic DNA and faster.

A total of 2910 random samples of blood (n=2064) and ticks (n=846) were tested with the pCS20 real-time PCR (TaqMan) diagnostic test. Of these only 88 blood samples (4, 2 %) and 254 tick samples (30 %) tested positive. Ten new isolates were identified and named after their place of origin: Rockhurst, Silverdale, Argyll, Glendowan, Thorndale, Claypits, Springbokfontein1 (SBF1), Springbokfontein2 (SBF2), Langverwacht and Klipplaatrief.

The pCS20 sequences of samples from 8 different farms were identical to that of the Rockhurst isolate. The sequence of Claypits was found on 2 different farms, the sequence of Mara 87/7 was found on 9 different farms, the sequence of kumm2 was found on 2 different farms, the sequence of the Welgevonden stock was found on 7 different farms and Blaauwkrans on 5 different farms in the endemic regions where samples were collected.

The two SBF samples were successfully established in sheep endothelial cells *in vitro*. The SBF1 and SBF2 isolates were used in a cross-protection study and only the SBF2 isolate protected the immunized sheep against a challenge with the Welgevonden stock.

Conclusions

In this study the identification of ten new isolates the Rockhurst, Silverdale, Argyll, Glendowan1, Thorndale, Claypits, Springbokfontein1 (SBF1), Springbokfontein2 (SBF2), Langverwacht and Klipplaatrif shows that genetic diversity of *E. ruminantium* isolates does exist in the field. The pCS20 sequence of these new isolates only differed by single nucleotide polymorphisms (SNPs) dispersed throughout the sequence. However, the pCS20 sequencing results of the Rockhurst isolate suggests recombination between the Blaauwkrans and Marra88/7 stocks.

The cross-protection tests undertaken between the well characterized 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 highlights the genetic diversity of isolates and may explain why only limited protection is obtained with the blood Ball3 vaccine in the field. Continual characterization of new isolates will benefit the development of an efficient vaccine against heartwater.

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