Journal of Animal and Veterinary Advances 5 (11): 990-994, 2006


# Molecular Characterisation of a HW 19 *Cowdria ruminantium* DNA Fragment for Development of Serological Assays Against Heartwater

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**Abstract:** A *Cowdria ruminantium* DNA fragment (4.2 Kb) cloned in a pGEM plasmid vector was 58 % sequenced, using a "primer walking strategy" in a thermo cycling silver sequencing technique. The aim of this experiment was to sequence the cloned insert and predict its immunogenic proteins by an *in vitro* transcription/translation assay. Incomplete nucleotide sequences of 1265 and 1201 base pairs in length were obtained, respectively from the 5' (T7 sequence) and 3' (Sp6 sequence) ends of the insert. These sequences were 70% A+T rich in composition. Homology search (BLAST) of the translated sequences against the genebank database identified significant similarities with two proteins of known functions from the T7 sequence and a non-specified cell division protein from the Sp6 sequence. Two major immunogenic proteins of 55 and 27 KDa were revealed by *in vitro* transcription/translation assay. Although the HW19 DNA fragment is incompletely sequenced, the two immunogenic proteins revealed may have a significant application for the development of improved serological diagnostic tests and/or subunit vaccine against heartwater.

**Key words:** Heartwater, silver sequencing, immunogenic proteins, subunit vaccine

## INTRODUCTION

The most significant tick-borne disease on the African continent in terms of economic losses and restriction of livestock development are Babesiosis, Anaplasmosis, Theileriosis and Cowdriosis or heartwater[1]. Since livestock remains a major source of nutritional proteins in most Third World countries, particular attention should be given to these diseases by developing sensitive diagnostic tests for detecting their presence or producing safe and protective vaccines against them.

Heartwater is an economically important infectious disease affecting domestic and wild ruminants[2]. The causative agent is a rickettsia[3], *Cowdria ruminantium*[4], which is transmitted by ticks, particularly of the genus *Amblyomma*[5]. In domestic ruminants, heartwater is characterised by high fever, anorexia, severe nervous symptoms and sudden death[6]. The disease is endemic in Africa south of Sahara and is widely spread in some Carribean Islands, where it causes about 90% mortality, thus contributing to a significant reduction of livestock production[7]. In Cameroon, the disease mostly occurs in the Northwest, West and Northern provinces. These regions are the main livestock producing areas in the country. In all the heartwater endemic areas, it is important to reduce losses due to mortality by regularly detecting and monitoring the disease. To date no specific sensitive serological diagnostic tests, or safe and protective vaccine has successfully been developed against heartwater. Diagnostic of heartwater by nucleic acid-based assays[8-10] still lack the sensitivity that is required at any time to detect carrier animals. Moreover, currently available serological assays using the immunodominant 32 Kda (MAP1) and 21 Kda (MAP2) proteins of *C. ruminantium*[11,12], give an unacceptable high number of false positives, due to cross reacting antibodies resulting from exposure to *Ehrlichia* species[13]. Although recent research has indicated the potential of MAP1 gene as a protective antigen[14], no heartwater vaccine is commercially available. Hence, there is still a need to investigate on immunogenic proteins for developing improved specific and sensitive serological assays or protective vaccine against heartwater.

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This study was carried out to investigate on a HW 19 *Cowdria ruminantium* DNA fragment by adopting a PCR-silver sequencing technique, analysing the generated sequence and predicting the molecular size of the immunogenic proteins encoded by the insert. The determination of the primary structure and further characterisation of these immunogenic proteins may have practical application in the development of improve diagnostic tests as well as subunit vaccines against the disease.

## MATERIALS AND METHODS

**Silver sequencing strategy:** Plasmid DNA used as template for sequencing was prepared from recombinant HW19 *Escherichia coli*[15]. The sequence of the HW 19 *Cowdria ruminantium* DNA was derived by using a silver sequencing method (Promega, Madisson, USA). Synthetic oligonucleotide primers were signed to extend the new strands from promoter T7 (5' end) and promoter Sp6 (3' end) sequences flanking the DNA insert within the template. Initial primers were designed from promoter T7 and Sp6 sequences. Further sets of primers (1, 2, 3, 4 and 5) were derived from subsequent sequences obtained. They were designed in the G+C rich region upstream the 3' end of previous sequences, leaving few nucleotides to identify overlaps with the generated sequences, by "primer walking strategy ".

**PCR-sequencing conditions:** Empirical optimisation of PCR reactions was conducted for annealing temperature at 45, 52 and 55°C and for two-fixed number of cycles (40 and 85). These conditions were applied for each set of primers, but in general, 52°C for 40 PCR cycles were maintained. PCR was performed for two sets of separated reactions A, C, G, T, using primers HW19-T7 and Sp6. A total of 16.2 μL per individual reaction contained 2 μL of each dNTP/ddNTP mix, 4.2 μL of enzyme/primer/template mix, layered with 10 μL of mineral oil to prevent evaporation. From one set of primers to another, the volume of DNA template/primer ratio was monitored to between 0.022 and 0.077. For a single PCR cycle, the Perkin Elmer Celtus thermocycle apparatus (Cetus corporation, USA) was programmed as follows: 2 min initial denaturation at 95°C in a boiling water bath; 30 seconds denaturation at 95 °C; PCR reactions annealing at 52°C and 1 min primer extension at 72°C. At the end of each cycle, reactions were further extended for 10 min at 72°C to allow their complete termination.

Amplified DNA fragments were separated on 6% (w/v) polyacrylamide gel. During separation, a temperature monitor (GIBCO, life technologies) was used to maintain the running temperature constant between 50 and 52°C. The silver staining for detection of resolved fragments consisted of three successive steps as follows: 25 min fixation; 45 min silver staining; and 10 min development of bands. The length of time separating silver staining of the gel, washing in ultrapure water and the contact with the developer was critical and so was optimised to take not more than 30 seconds. The stained DNA fragments were transferred in a dark room onto an automatic film processor. Nucleotide residues were read from the bottom to the top of the film in the 5' to 3' direction. The number of nucleotide residues generated from a single was obtained by locating and reading the sequence from overlapping region separating the previous and the new sequence.

**Nucleotide content analysis:** The G+C content of the generated sequence was analysed by a Dnasis/Genepro software computer program from the University of Zimbabwe. Homology analysis was performed at the University of Florida, USA, using BLAST files[16].

**Identification of immunogenic proteins:** An *in vitro* transcription/translation assay was carried out to predict the HW19 expressed products. The method is based on Promega protocol for the E. coli S30 extract system of circular DNA. Each of the HW19 and the control reactions were performed in a total volume of 50 μL containing 5 μL of amino acid mix minus methionine, 20 μL of S30 premix, 1 μL of $^{35}S$ methionine and 15 μL linear extract S30. The control reaction was completed to 50 μL with 3 μL of pGEM DNA (1.25 μg $\mu L^{-1}$) and 6 μL of distilled water, while 2 μL of DNA template (2.47 μg $\mu L^{-1}$) and 7 μL of distilled water were added to the HW19 reaction. The control (1 μL) and the HW19 (2.5 μL) reactions were centrifuged in 20 μL Acetone after 2 h incubation at 37°C in water bath. The pellet was dried, dissolved in sample buffer, denatured at 100°C in water bath and fractionated in 12% Acrylamide/2.7% Bis- acrylamide SDS-PAGE. Translated products were exposed for 2 days at –80°C onto a Kodak safety film.

## RESULTS AND DISCUSSIONS

**Optimal PCR-silver sequencing conditions:** Reproducible sequences were obtained after repeated experiments for the conditions shown in Table 1.

The DNA template/primer ratio was maintained between 0.02 and 0.07, but most primers work well for ratios between 0.02 and 0.05. A suitable ratio for reproducible sequence may depend on the nucleotide content and the length of the primer, but also on the

5' end origin

1

gatcctgctaatactttaggtaatgttttttaagatgtggcctagataaataaaacactgcttgactgtctatatagtacatacgtgatagataa
cgaaaaaaatttctcaaagcagacaccttgcattggtaactgcttcactccacattgatatctagaagcaaaccattttctcaaatctccaatttt
taacatgcacaagattatttaatgtaacagaacaccctgtactcttatgcaagaactctataaacttatcaaggtctcttacataagaaacaac
gtgtttcaaagaatacctcttctcttgttccatccatattttccagattatttacaatatcatagagttccttttattcgacatactattaaaatcaatc
aaaacaaaataatagtactatctaattaataaaaaaatcctcaatagtctttagcaagaactacaatataatgagacacttaatctattaatatat
ttcaagatttattatactactcatcatatttagcataatatcacatctgacatgattaacaatagtaacacatttcttaacacagataatatactaaa
tatatatttgctggtttcttgatataaattgaggtaaaaaatgaaaatgcagtattttgaggtgcaggactcatttggcacagcagatagcaga
ttgcagctgtcagcacatggtatatcagttggaacttatggggacgtgtacatagaaatattacacatatgaaacacttaccgaaaaaacttt
aaaatatttacccacatatcatctaccagacaacatatatgggaaccagcaatatagacgaagtattatctgacacatacatgtacttatctta
actattcctacacaaccaattgacggcacccgagtatgtacacaataccaagcacaaccagcatatgtgtaaaaaatactcaatattaatttg
tagtaaaggtatcgaaattacatcactcaaatttcccagtgaaatagcagaagaaatttacaatataatccaatttttatactctctggtccaag
ttttgctaaagaaattgcagaacatcttccttgtagtatagtacttgctggtgataataaagaacttggtgtaatcattagaaacaataagtaat
agtgttctaaaataatataccatcaagatattataggtgtacagttggagctgcattaaagaacataattgcaagttgcatgtggaataatcgc
tggaaaatttaggtaataat 1265

The T-7 primer sequences (1, 2, 3, 4, 5) from the top to the bottom are underlined. The overlapping regions extended by each primer are in bold. The initial T-7 primer sequence is not shown. The 5' end sequence starts with position 1.

Fig. 1: 5' proximal 1265 bp nucleotide sequence of the HW19 *C. ruminantium* DNA fragment

3' end origin

1

gatcaattagtcaattgtggatttactgttgataaaatattgattgatggtaatgaatatgtaagttctgatgaaatacgtaa
acttgttgatgcaaggtctatcttttttgtgcctttggctgatttgagaaacaaaatagagtctagtcatccatggatc
aaaaatgcctcagttaagaggctgttaccaaatactttacagataacagttcaaagaatattctgctttgcaaattggtatcatgataacaaa
aattctatcattgatagttttggacatattattgttgataattgtaatatacggggatttgacttcaatacatggagatggtgcgttaactcatct
agattttatacgtgaagtagtcaatgataatactcttgttggtggtatggtttc atctatctatcacatatgttgatagtcattggtgggatattattt
tgtctagtggtttaaatataaaattacctaataatgattcttatgctgccttggcgtgaattattgaatatctataaagcttcaagtgagacttta
gtatggaaaacatatagcatgcgtgttccaggaaggttaatataatgaaataacttttattaataattagttactatagggtttacaagttgatagt
aagttttattaagtgtttttattttgtttttggatataggaaacctatgttgataaattagtaataatagtaactaatttttataatatgtttaaagttta
taagtaaaaaataatgaatttaatatattggttgttgatcattagtgtgtgtggtagtattatgaggtgtactttaaaggagtacattgtgtttgca
gtatttcattagtattgtaggtaattagtggcacaggttttgaaatgtccaaggaaagattgtatattttgcgtaagtattagttgttgatgatgt
agttgatatcagaaatctaataaaagatatattaagtgatgataattatgtcactaaattagcagttgatggtttatccgcgactcagatggctt
atgaaaaagagcctatgttgtattattggatatatggcttaagagggatctgatacttgatggattaagtgtactggaaaagccttaaagaaa
ggtatccttatttgcctattattatgccattgtgggcatggtaatattgccactgctgtaaagtctcaattggtcctt 1201

The Sp6 primer sequences (1, 2, 3, 4, 5) from the top to the bottom are underlined. The overlapping regions extended by each primer are in bold. The initial Sp6 primer sequence is not shown. The 3' end sequence starts with position 1.

Fig. 2: 3' proximal 1201 bp nucleotide sequence of the HW19 *C. ruminantium* DNA fragment.

Table 1: Optimal silver sequencing conditions

| Parameters | Optimal conditions |
|---|---|
| Template/primer ratio | Between 0.022 and 0.05 |
| Long glass plate preparation | 1.5 mL Glacial Acetic Acid and 1µL Bind Silane |
| Short glass plate preparation | 0.5 mL of 95% ethanol and 0.5 mL Sigma Cote |
| Number of PCR cycle | 40 cycles |
| Annealing temperature | 4 to 9°C below the Tm of primers |
| Gel concentration | 6 % (w/v) SDS-PAGE |
| Running temperature | Constant between 50-52°C |
| Critical step silver staining | Maximum 30 sec |
| Film exposure time | Maximum 10 min |

ddNTPs/dNTPs which should be around 1/100[17]. Generation of gosh bands following inappropriate choice of numbers of PCR cycles has been attributed to inhibition of enzyme activity or mispriming[18]. Our results showed that the annealing temperature should be 4 and 9°C below the melting temperature of the primers. This finding correlates with suggestions that a good annealing temperature should be 5°C below the melting temperature of the primer[19]. The sequenced DNA fragments were separated on 6% (w/v) SDS-PAGE so a s to enhance the

resolution of longer DNA fragments[20]. Other optimal conditions were empirical and depended on the experimental conditions.

**The HW19 DNA sequence:** The partial assignment of nucleotide sequence of the HW19 DNA insert are demonstrated in Figures 1 and 2 and consisted of 1265 base pairs from the 5' end and 1201 base pairs from the 3' end of the insert. These sequences were generated from each end by five primers.

A good primer was judged by its ability to generate an overlapping region closer to its priming site. Primers Sp6-2, T7-3 and T7-4 were found to generate the highest nucleotide residues (321, 342, 338, respectively). This was slightly above the range of 150-300 nucleotide residues[21] and confirm the reliability of the experiment.

**Nucleotide content of the HW19 DNA sequence:** The partial sequence of the HW19 DNA insert was 30% G+C rich in content. The deduced A+T content, which was 70%, characterises microorganisms of the order *Rickettsiales*. The homology search using the partial HW19 DNA sequence against the genebank data base revealed a significant similarity of the T-7 sequence with Glyceraldehyde-3-phosphate deshydrogenase and integrase. The 3' end sequence showed significant homology with an unspecified cell division protein found in several bacteria. The low G+C content of the generated HW19 sequence correlates with previous reports obtained from other sequenced fragments of *C. ruminantium*: 70% A+T for PCS 20 DNA probe[8]; 70% A+T for MAP1 gene[22]; 74% A+T for MAP2 gene[9]; A+T for the 58 kA protein gene[23]. Apparently, rickettsia such as *C. ruminantium* is unable to synthesise NAD and must obtain it from the host[24]. The Glyceraldehyde-3-phosphate deshydrogenase revealed by homology search may help *C. ruminantium* to derive its NADH and hence, its NAD from the host, whereas the cell division protein may be involved in the active process of proliferation of the parasite[25].

**Immunogenic proteins encoded by the HW19 DNA insert:** The different immunogenic proteins detected are shown in Fig. 3. Two immunogenic proteins of 27 and 55 KDa seem to be encoded by the HW19 DNA insert (lane b). These proteins were not present in the pGEM control reaction (Figure 3, lane a). The HW19 DNA insert of 4.2 kb is long enough to code for the two immunogenic proteins expressed. These results suggest the probable presence of two open reading frames within the insert. An antigenically conserved 27 Kda

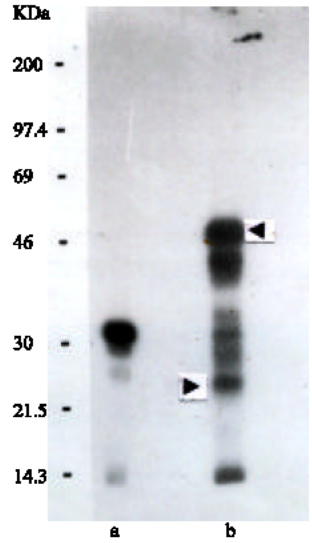


Fig. 3: Immunogenic protein patterns of the HW19 DNA insert: Lane a: plasmid pGEM or control proteins; Lane b: HW19 immunogenic proteins (◂ 55 KDa; ▸27 KDa)

protein of *C. ruminantium* was previously identified by immunoblotting with sheep and bovine antisera[26]. Similarities between the two 27 KDa proteins need to be established, for example by sequential precipitation method. The role of the potential proteins encoded by the complete sequence of the HW19 DNA fragment remains to be investigated.

## CONCLUSION

The fact that homology search did not reveal similarities between the HW19 of *C. ruminantium* DNA insert and closely related sp. such as *Escherichia* and *Anaplasma* (data not shown), eliminates the possibility of cross-reactivity with these species. Therefore, the DNA inserts coding for the two immunogenic proteins could be identified and probably used for the development of an improved serological diagnostic test or subunit vaccine against heartwater.

## ACKNOWLEDGEMENT

The authors are grateful to colleagues whose technical assistance was essential in the realisation of this study. The financial support of the Heartwater Research Project of the Veterinary Labolatory, Causeway-Harare, Zimbabwe is hereby acknowledged.

## REFERENCES


1. Mckeever, D.J., 1993. Progress towards a vaccine against *Theileria parva*. Reliance for heartwater research. Revue. Elev. Méd. Vét. Pays Trop., 46: 231-235.
2. Alexander, R.A., 1931. Heartwater. The present state of our knowledge of the disease. In 17th report of the director of veterinary services and animal industries, Union of South Africa, pp; 89-150.
3. Moshkvsky, S.D., 1947. Comments by readers. Sci., pp: 106: 62.
4. Cowdry, E.V., 1925. Study on the aetiology of Heartwater. Observation of a rickettsia, *Rickettsia ruminantium* in the tissues of infected animals. J. Exp. Med., 42: 131-252.
5. Uilenberg, G., 1983. Heartwater (*Cowdria ruminantium* infection). Current status. Adv. Vet. Sci., 15: 255-267.
6. Prozesky, L., 1987. The pathology of heartwater. A review. Onderstepoort J. Vet. Res., 54: 193-196.
7. Burridge, M.J., 1985. Heartwater invades the Caribbean. Parasitol. Today, 1: 175-177.
8. Waghela, S. D., F. R. Rurangirwa, S. M. Mahan, C. E. Yunken, T. B. Crowford, A. F. Bardet, M. J. Burridge and T. C. McGuire, 1991. A cloned DNA probe identified *Cowdria ruminantium* in *Amblyomma variegatum*. J. Clin. Microbiol., 29: 2571-2577.
9. Mahan, S.M., S.D. Waghela, P.C. McGuire, F.R. Rurangirwa, L.A. Wassink and A.F. Bardet, 1992. A cloned DNA probe for *Cowdria ruminantium* hybridises with eight Heartwater strains and detects infected sheep. J. Clin. Microbiol., 30: 981-986.
10. Peter, T., S.L. Deem, A.F. Bardet, R.A.I. Norval,B.H. Simbi, J.P. Kelly and S.M. Mahan, 1995. Development and evaluation of PCR assay for detection of low levels of *Cowdria ruminantium* infection and *Ambliomma variegatum*. J. Clin. Microbiol., 33: 166-172.
11. Mahan, S.M., N. Tebele, D. Mukcedeya, S. Semu, C.B. Nyathi, L.A. Wassink, J.P. Kelly, T. Peter and A.F. Bardet, 1993. An immunoblotting diagnostic assay for heartwater based on the immunodominant 32 KDa protein of *Cowdria ruminantium* detects false positive in field sera. J. Clin. Microbiol., 31:2729-2737.
12. Mahan, S.M., P.C. McGuire, M.V. Bowie, F. Jongejan, F.R. Rurangirwa and A.F. Bardet, 1994. Molecular cloning of a gene encoding 21 KDa protein of *Cowdria ruminantium*. Microbiol., 141: 2135-2142.
13. Jongejan, F., N. De Vries, J. Nieuwenhuijs, A.H.M. Van Vliet and L.A. Wassink, 1993. The immunodominant 32-kilodalton protein of *Cowdria ruminantium* is conserved within the genus Ehrlichia. Revue. Elev. Méd. Vét. Pays Trop., 46: 145-152.
14. Nyika, A., A.F. Barbet, M.J. Burridge and S.M. Mahan, 2002. DNA vaccination with the MAP 1 gene followed by protein boost augment protection against challenge with *Cowdria ruminatium*, the agent of heartwater. Vaccine, 20: 1215-1225.
15. QIAGEN plasmid handbook, 1993. QIAGEN, pp:12-13.
16. Gish, W. and D.J. State, 1993. Identification of protein coding regions by data base similarity search. National Genetic, 3: 266-272.
17. Hindley, J., 1988. DNA sequencing. Laboratory techniques in Biochemistry and Molecular Biology. Elsevier Biochemical Press, Amsterdam, New York, Oxford, 384 p.
18. Shigeru, T., K. Minorn and K. Motoya, 1993. Direct sequencing of PCR products using unlabelled primers. Biotechniques, 12: 218-221.
19. Zyskind, J.W. and S.I. Bernstein, 1992. Recombinant DNA. A laboratory manual. Academic Press, Inc. San Diego, New York, Boston, Sydney, Tokyo, Toronto, pp: 234.
20. Kaisser, R., T. Hubkapiller, C. Heiner and L. Hood, 1993. Specific primer-directed DNA sequencing analysis using automated fluorescent direction primers. Methods Enzymol., 218: 122-145.
21. Olstone, M.A.A., 1990. Animal virus pathogenesis. A practical approach. Erlpress, pp: 174.
22. Van Vliet, A.H.M., F. Jongejan, F. Van Kleef and B.A. M.Van Der Zeijst, 1994. Molecular cloning, sequence analysis and expression of the gene encoding immunodominant 32-KDa protein of *Cowdria ruminantium*. Infect. Immu., 62: 1451-1456.
23. Lally, N.C., S. Nicoll, E.A. Paxton, C.M. Cary and K.J. Sumption, 1995. The *Cowdria ruminantium* groE Operon. Microbiol., 241: 2091-2100.
24. Cano, R.J. and J.S. Colome, 1986. Microbiology. West Publishing Company, St. Paul, New York, Los Angeles, San Francisco, 888 p.
25. Brock, T.D. and M.T. Madigan, 1991. Biology of microorganisms. 6th Edn., Printice-Hall International, Inc. New York, London, pp: 847.
26. Rossouw, M., A. W.H. Neitz, D.J. De Waal, J.L. Du Plessis, L. Van Gas and S. Brett, 1990. Identification of the antigenic proteins of *Cowdria ruminantium*. Onderstepoort J. Vet. Res., 57: 215-221.