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GROWTH OF *COWDRIA RUMINANTIUM* IN TISSUE CULTURE ENDOTHELIAL CELL LINES FROM WILD AFRICAN MAMMALS

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ABSTRACT: Endothelial cell cultures were established from several wild African mammalian species. Long-term cultures were established from three ruminants, sable antelope (*Hippotragus niger*), buffalo (*Syncerus caffer*), and eland (*Tragelaphus oryx*), and from an omnivore, the bushpig (*Potamochoerus porcus*). *Cowdria ruminantium* was isolated from plasma of clinically affected animals in these four cell lines and in bovine endothelial cells used routinely for *C. ruminantium* propagation. Nineteen different strains of *C. ruminantium* from Africa and the Caribbean region were grown and maintained in these cell lines and their growth was comparable with growth in the bovine endothelial cells. The role of sable antelope, eland, and bushpigs in the epidemiology of heartwater is unknown. However, these results extend the number of cell lines that can be used to isolate and grow *C. ruminantium*.

Key words: Cowdria ruminantium, culture techniques, endothelial cells, heartwater, wildlife.

INTRODUCTION

The rickettsia *Cowdria ruminantium* is the causative agent of heartwater, one of the most important tick borne diseases of domestic ruminants in Africa (Uilenberg, 1983). An obligate intracellular pathogen, *C. ruminantium* has a predilection for endothelial cells, macrophages and neutrophils and can be propagated continuously *in vitro* in bovine, ovine, and caprine endothelial cells (Byrom and Yunker, 1990). It is transmitted by ticks of the genus *Amblyomma*, the major vectors being *A. hebraeum* and *A. variegatum* (Uilenberg, 1981). Animals born and reared in endemic areas are usually immune and carrier status has been demonstrated in cattle, sheep, buffalo (*Syncerus caffer*) (Andrew and Norval, 1989), blesbuck (*Damaliscus albifrons*) (Neitz, 1935, 1937), guinea fowl (*Numida meleagris*), and tortoise (*Geochelone pardalis*) (Oberem and Bezuidenhout, 1987) by tick transmission, and in black wildebeest (*Connochaetes gnu*) by blood transmission (Neitz, 1935). Tsessebe (*Damaliscus lunatus*), waterbuck (*Kobus ellipsiprymnus*) and impala (*Aepyceros melampus*) are suspected of being carriers of *C. ruminantium* because specific DNA has been detected by polymerase chain reaction in clinically normal free-ranging animals (Kock et al., 1995). Wild African animals appear to be tolerant of the disease (Oberem and Bezuidenhout, 1987) although clinical cases have been recorded in some species including springbuck (*Antidorcas marsupialis*) (Neitz, 1944), eland (*Tragelaphus oryx*) (Young and Basson, 1973), sitatunga (*Tragelaphus spekei*) (Okoh et al., 1987), lechwe (*Kobus leche kafuensis*) (Pandey et al., 1992), and steenbok (*Raphicerus campestris*) (Jackson and Andrew, 1994).

The expansion of game ranching in conjunction with cattle ranching, particularly in Zimbabwe and the Republic of South Africa, gives significance to the potential of wild animals as carriers of heartwater. Furthermore, translocation of endangered species to new areas for breeding and conservation has the potential of spreading the disease, even outside the African continent, if these animals are carriers of heartwater and a potential tick vector is present.

Greater clarification is needed in order to determine the status of heartwater in

African wild animals which are the hosts of both the tick vectors and the organism. *Amblyomma hebraeum* and *A. variegatum* feed on a wide range of wild animal hosts including reptiles, birds, and mammals (Norval, 1983; Petney et al., 1987). We determined the ability of various strains of *C. ruminantium* to invade, and grow in endothelial cells of wild African animals *in vitro* as a prelude to investigating the susceptibility of these species to *C. ruminantium* infections, and their possible role in the epidemiology of heartwater.

MATERIALS AND METHODS

Endothelial cells from wild mammals were isolated using the modified method of Hirumi and Hirumi (1984) as described by Byrom and Yunker (1990). Animals were not killed specifically for this study and material was obtained from animals dying of various causes. Isolation of endothelial cells was attempted from impala (*Aepyceros melampus*), side-striped jackal (*Canis adustus*), blue wildebeest (*Connochaetes taurinus*), bush baby (*Galago crassicaudata*), giraffe (*Giraffa camelopardalis*), sable antelope (*Hippotragus niger*), elephant (*Loxodonta africana*), bushpig (*Potamochoerus porcus*), buffalo (*Syncerus caffer*), eland (*Taurotragus oryx*), nyala (*Tragelaphus angasi*) and kudu (*Tragelaphus strepsiceros*).

Large blood vessels (either pulmonary artery or aorta) were collected aseptically in the field and placed in transport medium of phosphate-buffered saline (pH 7.4, lacking Ca^{++} and Mg^{++}) (PBS) containing penicillin-G (200 units/ml; CAPS (Pvt) Ltd., Harare, Zimbabwe), streptomycin sulfate (200 μg/ml; Sigma Chemical Company, St. Louis, Missouri, USA), neomycin sulfate (80 μg/ml; Sigma), tylosin tartrate (20 μg/ml; Sigma) and amphotericin-B (10 μg/ml; Sigma). When it was not possible to process the vessels immediately (due to collection from animals dying far from the laboratory) they were kept in transport medium on ice or refrigerated.

In the laboratory, vessels were washed with PBS and outer layers of connective tissue and fat were removed aseptically. The vessel lumen was kept wet and cut into pieces between 1 and 2 cm^2. These were placed lumen side down on drops of collagenase (1mg/ml in PBS; Sigma) in sterile Petri dishes and incubated for 30 min at 37 C. The endothelial cells were then detached from the vessel segments by washing with the collagenase/PBS from the incubation.

The cell suspension was transferred into sterile tubes and centrifuged at 200 × g for 5 min. Cells were resuspended and washed once in Glasgow modification of Eagle's minimal essential medium (GMEM) (Gibco BRL Products, Gaithersburg, Maryland, USA) supplemented with antibiotics as in the PBS, and 10% tryptose phosphate broth (TPB) (Sigma), before being finally resuspended in GMEM medium further supplemented with 20% fetal bovine serum (not inactivated) (FBS) (HyClone Laboratories, Logan, Utah, USA), 292 μg/ml L-glutamine (Sigma) and 300 μg/ml endothelial cell growth supplement (Sigma).

Endothelial cell suspensions were inoculated in 1 ml aliquots into 24-well tissue culture plates (Costar, Cambridge, Massachusetts, USA), incubated at 37 C in a humidified chamber and monitored for growth of cells. The cell suspension was not quantified. Fresh medium containing 25% filtered conditioned medium from established bovine endothelial cells was added to cultures which showed poor growth by day 14. After reaching confluency in the 24-well culture plate, the cells were subcultured into 25 cm^2 culture flasks and from there into larger flasks of 75 cm^2 and 162 cm^2 (Costar). Subsequent cell passages were carried out as described by Byrom and Yunker (1990) in GMEM growth medium, supplemented with 10% TPB, 10% FBS (not inactivated), L-glutamine (292 μg/ml), penicillin-G (100 units/ml), streptomycin sulphate (100 μg/ml), neomycin sulphate (40 μg/ml), tylosin tartrate (10 μg/ml) and amphotericin-B (5 μg/ml). Cultures were grown in closed flasks. Stocks of each cell line were stored in liquid nitrogen with 10% dimethyl sulfoxide (Sigma) by slow freezing for later use.

Cell lines were designated by the common name of the animal, the month, and last two digits of the year of isolation. The four cell lines showing most consistent growth were Bushpig 1194, isolated from the aorta of a young male animal; Sable 1194, isolated from the pulmonary artery of an adult female; Buffalo 595 and Eland 895 isolated from the aortas of adult female animals. The bushpig, sable and buffalo cells were harvested from the respective blood vessels 24 hr after the animals had died, and the eland cells were harvested from the aorta 4 days after death.

The bovine cell line routinely used at this time was BPA593/1 (Mahan et al., 1994) which had been isolated from the pulmonary artery of a freshly killed bovine from an abattoir. Human umbilical vein endothelial cells (HUVEC) were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA) and grown at the same time. All cell lines isolated were confirmed as endothelial cells by indirect

TABLE 1. Strains of *Cowdria ruminantium* from Africa used in this study.

Country	Strain	Origin/reference
Zimbabwe	Beatrice[a]	18°47′S, 30°40′E
Zimbabwe	Crystal Springs	Byrom and Yunker, 1990
Zimbabwe	Finale[a]	20°17′S, 30°11′E
Zimbabwe	Highway	Byrom and Yunker, 1990
Zimbabwe	Hunyani[a]	17°41′S, 30°14′E
Zimbabwe	Kwekwe[a]	19°12′S, 29°59′E
Zimbabwe	Lemco T3	Byrom and Yunker, 1990
Zimbabwe	Mbizi	Byrom et al., 1991
Zimbabwe	Mubayira[a]	18°19′S, 30°34′E
Zimbabwe	Palm River	Byrom and Yunker, 1990
Zimbabwe	Plumtree	Mahan et al., 1994; Deem et al., 1996
Zimbabwe	Rusape[a]	18°43′S, 32°28′E
Republic of South Africa	Ball 3	Haig, 1952
Republic of South Africa	Welgevonden	du Plessis, 1985
Kenya	Isiolo	Ngumi et al., 1997
Nigeria	Nigeria	Ilemobade and Leeflang, 1977
Tanzania	Tanga	Arusha[b]

[a] Isolated from *A. hebraeum* ticks collected at these locations.
[b] Isolated from *A. variegatum* ticks collected at Tanga, Tanzania.

fluorescent antibody testing for Factor VIII antigen (von Willebrandt's factor; Ryan, 1984).

Prior to infection with *C. ruminantium*, the medium on the endothelial cells was changed to Leibovitz L-15 medium (Gibco) supplemented with 0.45% glucose, 10% TPB, 5% FBS, (not inactivated), L-glutamine (292 μg/ml), penicillin-G (100 units/ml) and amphotericin-B (5 μg/ml) (Byrom and Yunker, 1990). Infection of cells was accomplished by two methods. The first was by inoculation of cells with plasma from febrile animals (sheep or goats) which had been experimentally infected with different strains of *C. ruminantium* (Byrom et al., 1991). Equal aliquots of 1 to 4 ml of plasma from individual animals were inoculated on to 4 to 12 flasks of different cell lines at the same time to allow for comparison between cell lines. There were more BPA593/1 flasks available as these were the cells used for routine propagation of *C. ruminantium* in the laboratory.

The second method of infection of cells was by inoculation of supernate, or scraped cells and supernate from *C. ruminantium*-infected bovine endothelial cell cultures (Byrom and Yunker, 1990). Inoculation of infected material on to a 25 cm^2 flask ranged from 1 ml of supernate from a strongly infected culture with many elementary bodies (EBs) and colonies present, to 4 ml of scraped cells and supernate from cultures with fewer EBs.

Wild animal cell lines Bushpig 1194, Sable 1194, Buffalo 595, Eland 895, Giraffe 295, Kudu 1294 and Bushpig 696; bovine endothelial cells, and HUVEC cells were infected with 19 different strains of *C. ruminantium*. These included 17 strains from Africa as listed in Table 1, and two from the Caribbean; Gardel (from Guadeloupe; Uilenberg et al., 1985) and Antigua (Birnie et al., 1985). In addition, for Crystal Springs two separate stocks were maintained. One of these, designated Crystal Springs High Passage, had been in culture for more than 2,000 days. It was kept separate from the other Crystal Springs stocks (passage levels up to 50) although no change had been observed in its characteristics. All newly isolated strains (those indicated by grid references in Table 1) were procured by collecting *A. hebraeum* ticks from the respective areas. The ticks were fed on susceptible sheep or goats and when these animals became clinically ill, their plasma was used to isolate new strains (Byrom et al., 1991).

Speed of infection and yield of *C. ruminantium* (Crystal Springs low passage) organisms grown in four different cell lines (BPA593/1, Sable 1194, Bushpig 1194, and Buffalo 595) were compared. Five 25 cm^2 flasks of each cell line were inoculated with 350,000 cells per flask and grown to confluency. *Cowdria ruminantium*-infective inoculum from culture material was inoculated on to the flasks. The inoculum was quantified by staining with 6-carboxyfluorescein diacetate (cFDA, Sigma) to determine viability (Mahan et al., 1995). Each flask was inoculated with 5.92×10^7 viable EBs or infective units of *C. ruminantium*. Flasks were monitored for cytopathic effect (CPE) and sampled at intervals of 2 to 4 days for in-

fection levels. When most of the endothelial cells had been lysed by infection four flasks of each group were harvested, and the total yield of *C. ruminantium* organisms per flask was measured by staining with acridine orange (Mahan et al., 1995). Yields from the endothelial cell lines of the different species were compared, and differences were analysed using the Newman-Keuls multiple comparison test (Anderson and McLean, 1974).

RESULTS

Long-term endothelial cell lines were successfully established and maintained from bushpig (Bushpig 1194 and Bushpig 696), sable antelope (Sable 1194), buffalo (Buffalo 595), and eland (Eland 895). Cell lines were initiated from giraffe (Giraffe 295) and kudu (Kudu 1294 and Kudu 595), but growth could only be maintained for four to seven passages. Cell lines successfully initiated from wildebeest (Wildebeest 795) and impala (Impala 695) were lost as a result of fungal contamination. Establishment of cell lines from elephant, bushbaby, nyala, and side-striped jackal were attempted without success. All successfully isolated cell lines showed the typical cobblestone morphology of endothelial cells and were confirmed to be of endothelial origin by Factor VIII staining. Stocks of each line were successfully frozen and retrieved from liquid nitrogen.

Although all cell lines were grown under the same conditions, not all grew at the same rate. Transformation was not observed in any of the cell lines and growth declined after a number of passages. The Bushpig 1194 cells were passaged first from the 24-well plate after 30 days and were grown through 22 passages before growth declined; Sable 1194 were first passaged after 28 days and then grown through 26 passages; Buffalo 595 cells grew vigorously and were first passaged after eight days and then grown through 26 passages before growth declined. Buffalo 595 cells grew to a greater density than the other cell lines with an average cell density of 2.5×10^5 cells per cm^2 of cell monolayer, while Bushpig 1194 and Sable 1194 grew to an average cell density of 9×10^4 cells per cm^2 of monolayer. Eland 895 cells also grew vigorously with an average cell density of 1.5×10^5 cells per cm^2 of monolayer, and were first passaged from the 24-well plate after 7 days and grown through 33 passages before growth declined. The bovine endothelial cell line BPA593/1 was grown through 60 passages before growth declined, with an average cell density of 1.5×10^5 cells per cm^2.

Results of inoculation of plasma on to wild animal endothelial cell cultures, and bovine endothelial cell cultures inoculated concurrently, are shown in Table 2.

Cowdria ruminantium isolation of Mbizi, Kwekwe and Rusape strains occurred first in Sable 1194 cells, and Nigeria D225 strain was only isolated in Sable 1194 cells. Similarly the Antigua strain was isolated first on Bushpig 1194 cells, and the Tanzania strain was only isolated on Bushpig 1194 cells. Thereafter, these strains were successfully passaged on to other cell lines.

Eighteen different strains of *C. ruminantium* were successfully propagated on wild animal endothelial cells from infected bovine cell culture material. All strains were routinely and consistently grown in bovine endothelial cells. The number of consecutive passages and the average passage interval of each strain in different animal cell lines are shown in Table 3. Different strains of *C. ruminantium* had different growth patterns and some were markedly easier to grow than others, as indicated by the differences in average passage times.

Growth of some strains appeared to be superior in wild animal cell lines. Sable 1194 infection with the Mbizi strain had a shorter average passage time over 23 passages, than that of any bovine cell line (Table 3).

Cowdria ruminantium also was grown on Kudu 1294, Giraffe 295, HUVEC and Bushpig 696 cell lines. Three flasks of Giraffe 295 endothelial cells were infected with Crystal Springs and Lemco T3 strains; seven flasks of Kudu 1294 cells

TABLE 2. Isolation of *Cowdria ruminantium* on wild animal endothelial cell lines from plasma of infected ruminants.

Strain of *C. ruminantium*	Endothelial cell lines				
	BPA593/1[a]	Bushpig 1194	Sable 1194	Buffalo 595	Eland 895
Antigua	7 (4)[b]	5 (4)	5 (0)	5 (0)	8 (0)
Beatrice	5 (5)	1 (0)	1 (0)	1 (1)	--
Crystal Springs	11 (11)	4 (3)	4 (2)	4 (2)	--
Finale	4 (4)	3 (3)	2 (2)	2 (1)	3 (2)
Hunyani	4 (4)	3 (3)	2 (2)	2 (2)	--
Isiolo	8 (8)	2 (2)	2 (1)	3 (1)	2 (2)
Kwekwe	2 (1)	--[c]	2 (2)	2 (0)	1 (0)
Mbizi	12 (7)	3 (0)	3 (1)	2 (0)	1 (0)
Mubayira	4 (3)	1 (1)	1 (1)	1 (1)	1 (1)
Nigeria D225	7 (0)	--	1 (1)	--	--
Plumtree	3 (3)	1 (1)	--	1 (1)	1 (1)
Rusape	2 (1)	2 (1)	2 (2)	2 (1)	2 (2)
Tanga	4 (1)	2 (2)	--	2 (0)	2 (0)
Welgevonden	1 (1)	2 (0)	2 (2)	1 (1)	1 (1)

[a] Bovine endothelial cell line.
[b] Number of flasks (number of positive flasks).
[c] -- = not done.

with Crystal Springs and Mbizi strains, and 14 flasks of Bushpig 696 cells with Crystal Springs, Finale, Highway, Mbizi and Mubayira strains. Two flasks of HUVEC cells were infected with Crystal Springs strain.

In the direct comparison of Crystal Springs strain grown in four cell lines, the Bushpig 1194 cells reached maximum infection on day 10, those grown in Buffalo 595 cells on day 14 and those grown in Sable 1194 and BPA593/1 cells on day 17. The mean yield of EBs from a flask of Sable 1194 cells was 2.72×10^8 (SD = 0.41) and that from Bushpig 1194 was 3.23×10^8 (SD = 0.65). These two values are not significantly different from each other. However, the yield from both lines was significantly lower ($P < 0.05$) than that from the BPA593/1 bovine cells. There was no significant difference in the yields from Buffalo 595 cells (4.10×10^8, SD = 0.93) and the bovine cells (5.10×10^8, SD = 0.96), or in the yield from the Buffalo 595 and Bushpig 1194 cells. However yields of EBs from Buffalo 595 and Sable 1194 were significantly different ($P < 0.05$).

DISCUSSION

Endothelial cells were successfully isolated from several wildlife species, and four cell lines were propagated on a long term basis. Fourteen strains of *C. ruminantium* from different geographical areas were isolated from plasma of infected sheep and goats, and 18 strains were routinely grown and maintained in these wild animal cell lines. The Crystal Springs High Passage strain which had been adapted to grow in bovine cells for more than 2,000 days was easily grown in wild animal cell lines indicating that these cells were physiologically similar to bovine endothelial cells. Reasons for the differing growth patterns of different strains of *C. ruminantium* were unclear, but do not appear to be related to geographic origins of the different strains.

Variation in total yield of EBs between the different cell lines could be due to a number of factors. The inoculum in this experiment came from a culture grown in bovine cells and therefore may have required some fine adaptation to the wild animal cells. Yields of EBs from buffalo

TABLE 3. Serial passage of different *Cowdria ruminantium* strains in bovine and wild animal endothelial cell lines.

Strain of *C. ruminantium*	Endothelial cell lines					
	Bovine cells[a]	BPA593/1	Bushpig 1194	Sable 1194	Buffalo 595	Eland 895
Antigua	21 (13.1)[b]	11 (11.8)	4 (13.3)	5 (11.0)	4 (20.3)	2 (13.5)
Ball 3	44 (10.5)	24 (10.1)	5 (12.0)	6 (10.5)	1 (21.0)	6 (12.3)
Beatrice	—[c]	16 (8.4)	5 (8.8)	11 (15.4)	6 (15.2)	5 (7.0)
Crystal Springs	50 (9.7)	43 (8.9)	3 (11.3)	4 (10.8)	1 (14.0)	5 (8.8)
Crys Spr High Passage	309 (7.6)	159 (7.3)	5 (10.8)	6 (11.7)	10 (6.8)	8 (7.6)
Finale	—	25 (7.7)	5 (7.0)	15 (10.9)	10 (7.3)	14 (11.6)
Gardel	50 (8.8)	26 (11.4)	6 (10.5)	10 (10.6)	5 (13.2)	8 (14.5)
Highway	21 (8.5)	10 (9.1)	4 (10.5)	5 (11.2)	4 (10.5)	5 (10.2)
Hunyani	—	4 (6.8)	5 (9.4)	6 (7.5)	5 (9.8)	5 (8.8)
Isiolo	—	15 (16.3)	6 (12.0)	7 (9.3)	4 (8.3)	6 (11.5)
Kwekwe	—	7 (16.6)	5 (7.6)	5 (7.6)	—	5 (13.2)
Lemco T3	33 (7.4)	28 (7.5)	4 (13.0)	5 (11.6)	1 (9.0)	1 (14.0)
Mbizi	50 (10.7)	35 (10.4)	3 (8.7)	23 (7.4)	5 (16.4)	5 (13.4)
Mubayira	—	6 (8.7)	4 (9.0)	5 (10.0)	5 (11.8)	5 (11.0)
Nigeria D225	47 (10.5)	13 (12.0)	5 (8.8)	13 (11.4)	1 (8.0)	4 (9.3)
Palm River	51 (8.1)	9 (12.7)	1 (7.0)	5 (8.4)	6 (14.7)	3 (16.3)
Plumtree	36 (8.9)	34 (7.9)	6 (11.3)	6 (11.7)	6 (11.5)	8 (8.8)
Rusape	—	4 (22.0)	2 (19.5)	7 (8.7)	—	3 (21.3)
Welgevonden	27 (8.9)	27 (8.9)	6 (14.7)	8 (10.8)	6 (9.5)	6 (13.7)

[a] Cultures grown in bovine cell lines other than BPA593/1 or a combination of two or more bovine cell lines.
[b] Number of passages in sequence (average days per passage).
[c] — = not attempted.

and bovine cells were similar, which could be due to the fact that these species are phylogenetically closer to each other than to either bushpig or sable. The greater number of cells available for infection in a confluent flask of Buffalo 595 cells (with a higher cell density than the other two species) may simply have facilitated growth of larger numbers of *C. ruminantium* organisms. Despite lower yields of EBs in this comparison, *C. ruminantium* growth was not hampered in sable or bushpig cells.

Growth of *C. ruminantium* in human cells has been reported previously (Totté et al., 1993) and repeated in this study. *Cowdria ruminantium* was successfully grown in giraffe and kudu cells as well. However, susceptibility of these species to *C. ruminantium* infection has not been reported. This may suggest that host susceptibility to infection is related to factors other than the susceptibility of cultured endothelial cells to *C. ruminantium* growth.

These results extend the range of cell lines in which *C. ruminantium* has been grown. Although no correlation between *C. ruminantium* growth *in vitro*, and wild animal species as reservoir hosts for heartwater can be made from these studies, it nevertheless opens discussion on their possible role in the epidemiology of heartwater. This is more likely if these species occur in heartwater endemic regions and if *Amblyomma* ticks are known to feed on them. Bushpig and sable antelope are not known to be susceptible to heartwater although both occur in heartwater-endemic areas of Zimbabwe. Bushpig are likely to be hosts of *Amblyomma* ticks since the closely related warthog (*Phacochoerus aethiopicus*) is a known host of *A. hebraeum* (Norval, 1983). Sable antelope are hosts to ticks including *A. hebraeum* (Grobler, 1981) and are considered to be the most tick-attractive wild ungulate after eland (Lightfoot and Norval, 1981). The apparent greater efficiency of growth and isolation of *C. ruminantium* in the sable and bushpig cells justifies further investigation into the susceptibility of these species to

infection with *C. ruminantium*. Significantly it also provides options for isolation of the more fastidious strains of *C. ruminantium* in wild animal cells instead of bovine cells.

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