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Isolation of a Previously Undescribed Rickettsia from an Aborted Bovine Fetus

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A previously undescribed obligate intracellular bacterium was isolated from an aborted bovine fetus. The organism was resistant to penicillin, replicated within cytoplasmic vacuoles, exhibited structural characteristics compatible with the rickettsias, and shared antigenic determinants with *Cowdria ruminantium*.

Rickettsial pathogens induce diseases of the lymphoreticular, hemopoietic, vascular, digestive, and (less commonly) reproductive systems. Among members of the *Ehrlichieae* tribe of the rickettsias are agents which cause in domestic animals a number of serious diseases not known to be present in the United States. In Europe, the agent of tick-borne fever [*Ehrlichia* (*Cytoecetes*) *phagocytophila*] causes both systemic and reproductive diseases in sheep and cattle (1, 12). Another important exotic member of the *Ehrlichieae* tribe is *Cowdria ruminantium*, which causes serious losses in domestic livestock in Africa (6).

Abortions and stillbirths constitute serious losses to the U.S. cattle industry. The etiology, however, of most cases of bovine abortion submitted to diagnostic laboratories remains undetermined (3), suggesting a large reservoir of undetected abortigenic infectious agents. We report here the isolation from an aborted bovine fetus of an organism that appears to be a previously unreported rickettsia. It appears to be associated with reproductive failure in cattle, to be a member of the *Ehrlichieae* tribe, and to be antigenically related to *C. ruminantium*.

A 19-month-old primigravid Holstein aborted in month 3 of gestation. It was one of seven abortions during a 6-month period in a herd of 50 cows located in King County, Wash. Fetal liver and lung samples, placenta, and a vaginal swab from the dam were submitted refrigerated but unfrozen to the diagnostic laboratory.

The agent (WSU 86-1044) was isolated in bovine turbinate cells (BT cells; ATCC CRL-1390) cultured at 35°C in Eagle minimal essential medium with 10% fetal bovine serum, 100 U of penicillin per ml, 100 mg of streptomycin per ml, 50 μg of gentamycin sulfate per ml, and 1.25 mg of amphotericin B per ml. Homogenized and clarified lung and liver pools were adsorbed onto confluent monolayers for 1 h. Following isolation, infectivity titrations were performed on BT cells in 96-well plates. Tenfold dilutions in growth medium were mixed with cells and planted into 16 replicate wells per dilution. The plates were examined for cytopathic effects, and 50% tissue culture infective doses ($TCID_{50}$s) were calculated (7).

Growth rate, essential lipids, and cryostability. To examine the rate of replication, BT cells in 24-well plates were inoculated with 50 $TCID_{50}$s. Two wells were harvested at 4- to 24-h intervals and stored at −86°C until titration in duplicate. For essential lipids, infected medium was agitated with an equal volume of chloroform for 10 min at 23°C, and the aqueous phase was collected and tested for surviving infectivity. To assess the effects of various media on the loss of infectivity during freezing, duplicate samples of an unclarified pool were titrated in duplicate before and after one freeze-thaw cycle at −86°C in the presence of equal volumes of minimal essential medium–10% fetal bovine serum, minimal essential medium–10% fetal bovine serum–10% dimethyl sulfoxide, and Snyder II diluent with 1% bovine serum albumin.

Morphology. Infected and control BT cells on cover slips were infected and fixed 72 h postinoculation (p.i.) and stained with Giménez, Giemsa, periodic acid-Schiff, Brown-Brenn, Kinyoun acid-fast, and hematoxylin and eosin stains by standard procedures. For electron microscopy, infected cells were scraped manually from the surface at 72 h p.i. at about 75% cytopathic effect, pelleted and fixed in glutaraldehyde, postfixed, stained in block, embedded, sectioned, and stained with uranyl acetate by standard procedures.

Other agents. Infected culture material was screened for viruses by serial passaging in bovine cells and staining by indirect immunofluorescence (IFA) with a panel of antisera against common bovine viruses. For *Chlamydia* sp. testing, the isolate was centrifuged onto McCoy cell monolayers (ATCC CRL-1696) by standard procedures (10). Cultures were examined by direct immunofluorescence with defined chlamydial antisera. To attempt to isolate free-living bacteria, samples were cultured under aerobic, microaerophilic, and anerobic conditions in thioglycolate and on blood agar and chocolate blood agar. Attempts were made to isolate mycoplasma and L forms on modified Hayflick medium, Frey M96 medium, and tryptose serum agar supplemented with progesterone, testosterone, and diethylstilbestrol.

Immunofluorescence. Two yearling Nubian goats were inoculated intravenously and intraperitoneally with the isolate, one with 10^6 and one with 10^8 $TCID_{50}$/ml, and sera were collected 3 weeks p.i. for IFA staining. Infected and control BT cells on cover slips were fixed for 10 min in −20°C acetone and stored at −20°C. Cross-reactions with various members of the order *Rickettsiales* were evaluated by IFA with defined antisera on fixed BT cells infected with WSU

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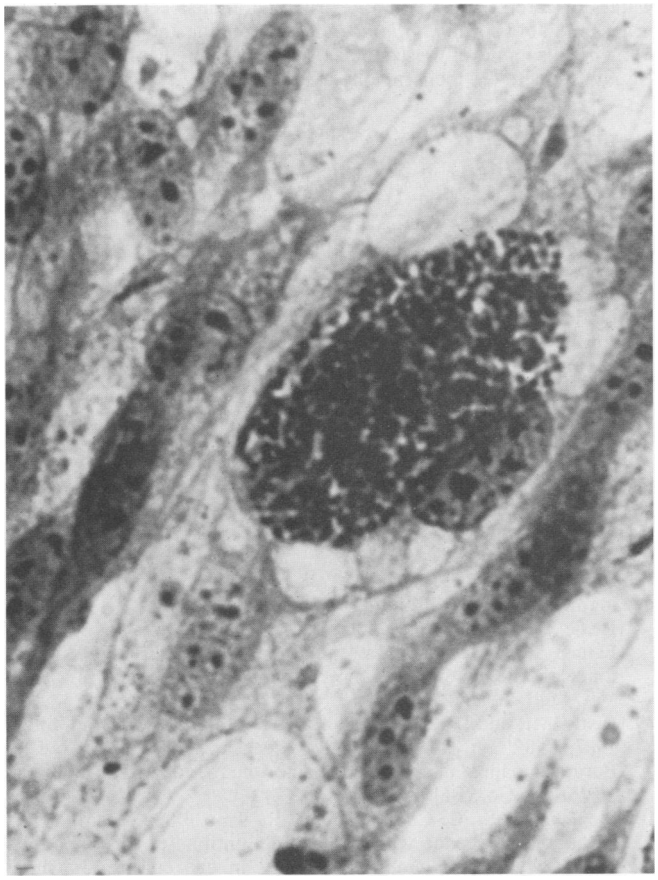

FIG. 1. Rickettsial isolate WSU 86-1044 in BT cells in vitro: large intracytoplasmic inclusion containing individual organisms (Giménez stain).

86-1044. IFA for *C. ruminantium* cross-reactivity was performed with the Kiswani strain of *C. ruminantium* grown in a calf endothelial cell line (13) and antiserum from a goat experimentally infected with the same organism.

Cytopathic effects appeared on the initial passage 36 h p.i. Multiple cytoplasmic vacuoles and inclusions were visible in stained infected cells (Fig. 1). The inclusions were gram negative, periodic acid-Schiff negative, and non-acid fast. During electron microscopy, numerous rickettsialike coccoid organisms were observed in membrane-lined cytoplasmic vacuoles. They divided by binary fission and had distinct plasma membranes and rippled outer cell walls with occasional surface blebs. There was considerable heterogeneity in staining densities and organism sizes (ca. 0.2 to 0.5 mm) (Fig. 2).

Initially, the isolate replicated in the presence of penicillin and streptomycin, which were removed after passage 3. Following experimental infection, the titer rose from $<10^{2.6}$ to $10^{8.5}$ $TCID_{50}$/ml by 96 h p.i. At this time, the monolayers were still adherent, cytopathic effects were prominent, and inclusions were present in approximately 80% of the cells. Infectivity was abolished by chloroform treatment. Freezing reduced infectivity only slightly: losses of no more than 0.3 $\log_{10}$ $TCID_{50}$ were observed with any of the three media tested. Infected cultures were found to be negative for bovine viral diarrhea virus, coronavirus, herpesvirus 1, herpesvirus 4, parainfluenza virus type 3, enterovirus, *Chlamydia* spp., free-living bacteria, mycoplasma, and L forms.

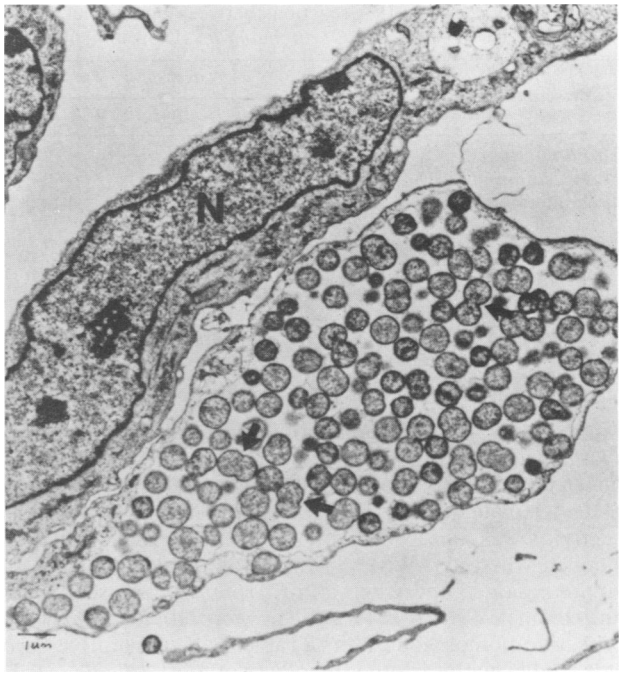

FIG. 2. Electron micrograph of organisms 48 h p.i. in a membrane-bound vesicle in the cytoplasm. The organisms vary in size and staining density. Organisms undergoing binary fission are indicated by arrows. N, Cell nucleus. Magnification, ×3,500.

IFA with WSU 86-1044-immune goat serum revealed specific fluorescence in prominent masses limited to the cytoplasm. Antibody (IFA) titers increased from preinoculation levels of 1:40 to 1:1,280 by 3 weeks p.i. The intravacuolar replication and ultrastructural appearance of the agent were compatible with those of members of the *Ehrlichieae* tribe (9).

TABLE 1. Organisms exhibiting negative serologic cross-reactions with bovine rickettsial isolate WSU 86-1044

Antiserum[a] (antigenic type)	Performing laboratory[b]
Rickettsia tsutsugamushi (Kato)	1
R. tsutsugamushi (Carp)	1
R. tsutsugamushi (Gilliam)	1
R. prowazekii	1, 2
R. akari	1, 2
R. conorii	1
R. canada	2
R. typhi	1
R. rickettsii	1
R. montana	2
R. rhipicephali	2
Coxiella burnetii phases I and II	1, 2, 3, 4
Wolbachia persica	2
Chlamydia psittaci	3, 4, 5
Chlamydia variant TWAR	5
C. trachomatis	5
Anaplasma marginale	4
Rochalimaea quintana	1

[a] Each yielded an IFA titer below the usual thresholds (range, 1:10 to 1:40) of the collaborating laboratories.

[b] Laboratories: 1, University of Illinois, Urbana, C. Holland (8); 2, Rocky Mountain Laboratory, Hamilton, Mont., W. Burgdorfer (5); 3, National Veterinary Services Laboratories, Ames, Iowa; 4, Washington Animal Disease Diagnostic Laboratory, Pullman; 5, University of Washington School of Public Health, Seattle, Cho-Chuo Kuo (10).

TABLE 2. Reciprocal IFA reactions between bovine rickettsial isolate WSU 86-1044 and *C. ruminantium* (Kiswani isolate)

Antiserum	Titer[a] on:	
	C. ruminantium	WSU 86-1044
C. ruminantium		
Preinfection	100	80
Postinfection	8,000	1,600
WSU 86-1044		
Preinfection	<50	<50
Postinfection	1,600	3,200

[a] Reciprocal of the highest positive dilution. Titrations were performed at The Ministry of Agricultural and Livestock Development, Kabete, Kenya.

Reference antisera against *Coxiella*, *Wolbachia*, *Chlamydia*, and *Anaplasma* spp. and 14 members of the genus *Rickettsia* all failed to react significantly with the WSU 86-1044 isolate (Table 1). However, a substantial cross-reactivity was observed with *C. ruminantium*. The titers obtained when anti-WSU 86-1044 was titrated on *C. ruminantium* antigen and when anti-*C. ruminantium* serum was titrated on WSU 86-1044 antigen were 20 to 50% of the respective homologous titers (Table 2). Comparative serologic studies with the other members of the *Ehrlichieae* tribe are in progress.

Antigenic cross-reactions in IFA between *C. ruminantium* and several ehrlichial agents have been reported (2, 4). The potential impact of *Cowdria*-related organisms within the United States on the IFA-based federal importation screening programs for heartwater is of considerable concern (4). *C. ruminantium*, being present in the Caribbean (11), represents an ever-present threat to the U.S. cattle industry. The existence in U.S. cattle of an organism that not only is a putative reproductive pathogen but also cross-reacts with *C. ruminantium* in the IFA test lends some urgency to the need for further studies on this organism.

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