Table 2. Cross -reactivity of hantavirus rN proteins with sera of rN-immunized rabbits

Serum of rabbit immunized with rN of	rN antigens used for the detection of lgG antibodies with ELISA				
	SEOV	HTNV	PUUV-Sot	SNV	ANDV
SEOV	**3,300,000**	1,600,000	76,800	38,400	51,200
HTNV	820,000	**820,000**	204,800	76,800	102,400
PUUV-Sot	6,600,000	6,600,000	**6,600,000**	3,300,000	6,600,000
SNV	3,300,000	3,300,000	13,200,000	**26,400,000**	13,200,000
ANDV	1,600,000	1,600,000	13,200,000	26,400,000	**52,800,000**

Given are the reciprocal endpoint titers. The homologous titers are shown in bold.

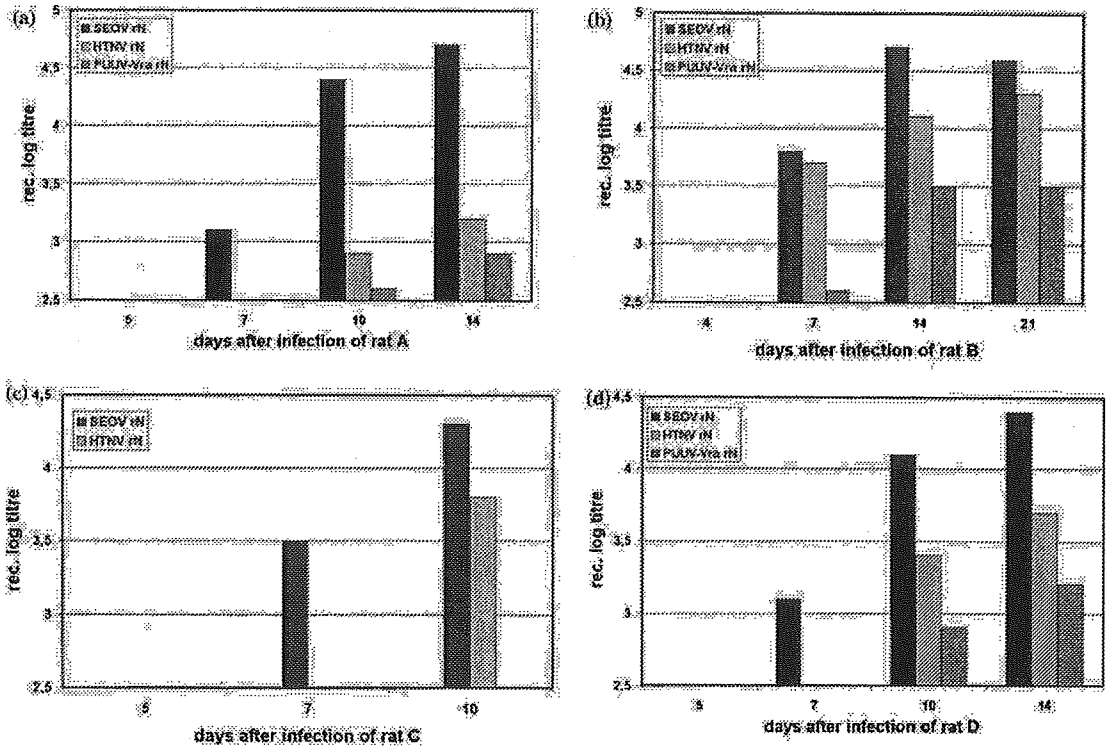

Fig. 2. ELISA reactivity of serial follow-up serum samples of four experimentally SEOV-infected Wistar rats using yeast-expressed rN proteins of SEOV, HTNV and PUUV-Vra. Microtiter plates were coated with rN proteins of SEOV (strain 80-39-B), HTNV (strain Fojnica) or PUUV (strain Vranica/Hällnäs). Thereafter, rat sera diluted serially two fold with an initial dilution of 1/200 were added. After incubation with HRP-labelled anti-rat IgG conjugate the immune reaction was visualized by addition of TMB substrate. Given are the reciprocal endpoint titers. The initial reciprocal log titer of 2.5 corresponds to a dilution of 1/400. As negative control, a serum from a wild-trapped rat from Japan, previously demonstrated to be non-infected [31], was used.

respective homologous antigens were found again to be the highest (Table 3). Thus, the anti-SEOV-positive serum reacted to the homologous SEOV rN antigen with the highest endpoint titer. As expected, its reactivity with HTNV rN was lower and the serum failed to react with rN proteins of the more distantly related PUUV, SNV and ANDV.

In contrast anti-HTNV- and anti-DOBV-positive serum pools reacted to equal endpoint titers to

Table 3. Cross-reactivity of hantavirus rN proteins with serum pools of HFRS patients infected with SEOV, HTNV, DOBV or PUUV and HCPS patients infected with SNV or ANDV, respectively

Human sera or serum pools	rN antigens used for the detection of IgG antibodies with ELISA				
	SEOV rN	HTNV rN	PUUV-Vra rN	SNV rN	ANDV rN
Anti-SEOV	25,600	4,800	<400	<400	<400
Anti-HTNV	51,200	51,200	2,400	2,400	3,200
Anti-DOBV	25,600	25,600	1,600	<400	<400
Anti-PUUV	9,600	1,600	25,600	12,800	12,800
Anti-SNV	<400	<400	4,800	51,200	12,800
Anti-ANDV	6,400	1,600	12,800	12,800	51,200

Given are the reciprocal endpoint titers. The highest titers are given in bold.

both SEOV and HTNV rN proteins. Pools of anti-PUUV and anti-ANDV-positive sera were found to cross-react also with SEOV rN protein, whereas the anti-SNV-positive pool failed to react with SEOV rN protein (Table 3). Interestingly, the level of cross-reactivity of the anti-PUUV- and anti-ANDV-positive pools to the HTNV rN protein was lower compared to that to SEOV rN antigen; the anti-SNV serum pool failed to react with HTNV rN protein. This difference in the cross-reactivity of anti-ANDV- and anti-SNV-positive serum pools with SEOV rN protein might be due to the fact that the anti-SNV-positive serum pool contained only acute phase sera whereas the anti-ANDV serum pool contained also late, convalescent sera.

Because of a previous report about a high seroprevalence of rats in Germany for SEOV [21] we performed an initial study on a small number of sera from wild rats (n = 11). These rats stemming from farms in Westphalia/Germany belonged to the species *R. norvegicus* as evidenced by the identity of the mt 12S rDNA sequence of liver tissue samples of two rats with that of an already published *R. norvegicus* sequence from Denmark (accession number AJ 428514; data not shown) and its divergency from the corresponding sequence of *R. rattus* (accession number AJ005780). As no SEOV-specific antibodies could be detected in this small number of wild-trapped rats (data not shown), additional studies are needed to prove the hantavirus seroprevalence of rats in Germany.

In conclusion, the N protein-encoding sequence of SEOV strain 80-39-B was expressed to high level in yeast *S. cerevisiae*. Initial studies suggest the usefulness of the antigen for the establishment of ELISAs to detect SEOV infections in rat and human sera.

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A pilot study for serological evidence of hantavirus infection in human population in south India

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***Background & objectives*: Hantaviruses are rodent-borne viruses of the family *Bunyaviridae* that have been identified as aetiological agents of two human diseases, haemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). There are no reports of hantavirus infections in humans from India, hence this pilot study was undertaken to provide the serological evidence of hantavirus infections in humans in south India.**

***Methods*: Serum samples were obtained from individuals with acute febrile illness and from voluntary blood donors, majority of whom were from south India. Serum samples were tested for anti-hantavirus IgM using a commercial enzyme immunoassay (EIA). Samples found positive by the EIA were tested by an indirect immunofluorescence assay (IFA) using slides coated with Seoul virus (SEOV) infected cells as substrate.**

***Results*: Of the 152 serum samples from individuals with pyrexic illness, 23 (14.7%) were positive for anti-hantavirus IgM by EIA. In contrast, only 5.7 per cent of healthy blood donors were positive by this assay. Eighteen of the 22 (82%) EIA-positive samples from patients were positive by the IFA assay. In contrast, only 2 of the 5 (40%) blood donor EIA positive samples were positive in the IFA assay.**

***Interpretation & conclusion*: The finding of this study indicated the possible presence of hantavirus infections in the human population of India presenting both as asymptomatic and symptomatic infections. Further studies need to be done to confirm the findings on a larger sample using molecular techniques.**

Key words Enzyme immunoassay - hantavirus - IgM antibodies - pilot study

Hantaviruses are enveloped viruses with a negative-sense single stranded RNA genome and belong to the family *Bunyaviridae*[1]. The spectrum of clinical symptoms caused by hantaviruses in humans varies from sub-clinical presentation to severe haemorrhagic fever with renal syndrome (HFRS) or pulmonary syndrome (HPS). Several genotypes/serotypes have been described of which at least five are pathogenic to humans[2]. The vast majority of human hantavirus infections are asymptomatic[3].

The natural reservoirs of hantaviruses are small rodents and transmission to man is believed to occur via aerosolized excretions. The worldwide distribution of rodents known to harbour hantaviruses suggests great disease causing potential[4].

The clinical diagnosis of hantavirus infections has routinely been confirmed by immunofluorescence antibody assay (IFA) or enzyme-linked immunosorbent assays (ELISA)[5]. The presence of cross reacting antibodies makes it difficult to distinguish hantavirus species by serology. Molecular tests based on nested reverse transcriptase-polymerase chain reaction (RT-PCR) have been used for diagnosis. Primary isolation of hantaviruses is most often attempted using Vero E6 cell line in laboratories with biosafety level-3 (BSL-3) facilities[6].

Hantavirus nucleocapsid protein (N) antigen elicits a strong humoral response in infected patients and immunized animals. High levels of antibody to the N antigen have been detected which indicated that it could be suitable as the sole antigen for serodiagnosis[7].

The Thottapalayam virus, which belongs to the same family, was isolated from the spleen of a shrew captured in July 1964, in Vellore, North Arcot district, Tamil Nadu, India[8]. Subsequently there have been no studies showing hantavirus infections in India. We undertook this study to investigate for the serological evidence of hantavirus infections in the human population by detecting anti-hantavirus IgM antibodies.

Material & Methods

Blood samples were obtained from 152 individuals who had acute febrile illness (duration <14 days). These patients were categorized into three different categories: (*i*) patients (n=67) with dengue-like illness but negative for dengue serology by a commercial Dengue Duo IgM and IgG Rapid Strip test[9]; (*ii*) patients (n=51) with leptospirosis-like illness, but negative for leptospira serology by the microscopic agglutination test (MAT)[10]. These patients were included because the early signs and symptoms of hantavirus infections mimic that of dengue and leptospirosis[11]; and (*iii*) patients (n=34) with suspected hantavirus infection. The samples were included by convenient sampling and had been submitted to the laboratory for various tests requested by the clinicians. Serum samples were stored at -20°C until testing. All the tests were conducted at the Department of Clinical Virology, Christian Medical College, Vellore. Since this was a pilot study, sample size was not calculated. All these patients presented with complaints of high grade fever with chills and rigors of <14 days duration associated with myalgia, headache and haemorrhagic manifestations like petechiae and purpuric skin rash.

In addition, 87 blood samples from voluntary blood donors were used as controls for the study. Sixteen samples were also included from patients with other viral infections as shown by corresponding IgM testing including dengue (n=10) and cytomegalovirus (n=6) (by a commercial Euroimmun kit, Germany) to serve as disease controls. All study samples were collected during a period of 15 months (July 2002 to September 2003). This study protocol was approved by the institutional research committee for scientific content and ethics.

The hantavirus IgM ELISA kit was procured from Focus Technologies (USA). This kit has been certified by ISO for human testing. This is an indirect antibody detection system; the assays were performed according to the manufacturer's instructions. As per the manufacturer's claim, this assay detects IgM antibodies against most of the known hantavirus subtypes including Puumala (PUUV), Hantaan (HNTV), Seoul (SEOV), Sin Nombre (SNV) and Dobrava (DOBV).

Briefly, microwell plates were coated with a mixture of baculovirus-derived recombinant nucleocapsid proteins from hantavirus strains. Serum samples (1:101 dilution) were incubated for 60 min, and after washing, anti-human IgM-horse radish peroxidase conjugate was added to the wells and incubated for 30 min. Tetramethylbenzidine was used as a substrate. After adding the stop solution, the resultant colour change was quantified by an EIA reader ELx 800 (Bio-Tek Instruments Inc. Vermont, USA). The optical density (OD) is shown to be directly proportional to the amount of antigen- specific IgM present in the sample. Sample OD readings were compared with reference cut off OD readings. Results

were reported as index values relative to the 'cut off' calibrator. To calculate the index values, specimen OD values were divided by the mean of the 'cut off' calibrator OD values.

All the samples found positive by ELISA were further tested by an indirect immunofluorescence assay (IFA) using Seoul virus (SEOV) (strain SR-11) infected Vero E6 cells as substrate. The serum samples were tested at a dilution of 1:10. Serum samples (15 µl) were added onto the 24 well slides and incubated for 30 min. The slides were then washed three times with phosphate-buffered saline (PBS, *p*H=7.4) and incubated with polyclonal rabbit anti-human IgM FITC-labeled conjugate (Dakocytomation) for 30 min. The slides were then washed and after mounting it with buffered alkaline glycerol, were read under the fluorescence microscope. All spots wherein 50 per cent of infected cells showing a characteristic apple green cytoplasmic fluorescence were recorded as reactive. Samples showing a positive reaction at 1:10 dilution were further diluted and tested at dilutions 1:20, 1:40, 1:80.

Data were analysed by Chi-square test using the EPI INFO (Version 6.04b), $P<0.05$ was considered significant.

Table I. Samples positive by ELISA and immunofluorescence assay (IFA)

Status of sample	No. positive by IgM ELISA	No. (%) positive by IFA
Samples from suspected hantavirus cases	22	18 (86)*
Dengue positive sample	1	0
Voluntary blood donors	5	2 (40)

*Only 21 samples tested

Table II. Clinical and laboratory findings in individuals with pyrexia of unknown origin and positive for hantavirus IgM antibody (n=12)

Clinical signs and symptoms	No. (%) positive
Fever	12 (100)
Nausea and vomiting	7 (58)
Hepatomegaly	5 (42)
Myalgia and headache	5 (42)
Cough	4 (33)
Dyspnoea	2 (17)
Urinary symptoms*	2 (17)
Rash	1 (8)
Laboratory findings	*No. (%)*
Thrombocytopaenia**	8 (67)
Leucocytosis***	7 (58)
Elevated liver enzymes+	3 (25)

*Increased frequency of micturition, dysuria, flank pains and haematuria

<100000 cells/mm^3, * >9500 cells/mm^3
+SGPT, SGOT > 100 U/ml, Alkaline phosphatase

Results

A total of 255 serum samples were tested for hantavirus IgM by ELISA; 28 (10.98%) of these were positive for hantavirus IgM by EIA (Table I). Twenty seven (96.4%) of the 28 positive samples were retested and produced reproducible results; one sample was insufficient for retesting. Ten of 67 (14.9%) samples negative for dengue, 9 of 51 (17.6%) negative for leptospirosis, 3 of 34 (8.8%) of those with suspected clinical hantavirus infection, and 1 of 10 positive for dengue infection, were hantavirus IgM EIA positive. Of the 87 voluntary blood donors, 5 (5.7%) were hantavirus IgM EIA positive. None of the six samples positive for CMV serology was hantavirus IgM EIA positive.

Of the 28 hantavirus IgM positive samples, only 27 could be tested by the IFA. One sample was insufficient for testing. Of these, 20 (71.4%) were also positive by IFA. A representative number (n=5) of EIA negative samples were negative by IFA.

Examination of the patients' clinical records revealed that of the 23 patients who were seropositive for hantavirus IgM, eight had other underlying diseases. Records of three patients were not available for evaluation. The remaining 12 labeled as cases of pyrexia of unknown origin (PUO) were evaluated based on the clinical history at the time of presentation, the laboratory findings and the final diagnosis (Table II).

Among the individuals with febrile illnesses (n=152, 59.6%), 14.5 per cent (95% CI: 9.5-20.75) were positive for anti-hantavirus IgM antibodies. The difference in the rate of positives between the study group and the blood donor healthy control group (n=87)

where the positivity rate was 5.7 per cent (95% CI: 2.13-12.27) was statistically significant ($P<0.05$).

Discussion

This is probably the first study in India showing serological evidence of hantavirus infection in the Indian population. Clinically, the febrile illness produced by dengue virus, leptospira serotypes and hantaviruses may be indistinguishable[11]. The rate of IgM positive status in the febrile illness group was significantly higher than that seen in the healthy blood donors. We also found that this test did not pick up significant false positives in the samples positive for dengue or CMV IgM. It could be assumed that the positives in the control group indicated asymptomatic infections. Likewise, the hantavirus IgM seropositives in patients with other underlying diseases could be due to serological cross reactivity or asymptomatic infections. Alternatively, it could also be speculated that there are multiple hantaviruses in circulation and that at least one is of low pathogenicity.

It was noted that the mean of index values of the blood donor controls which were positive for hantavirus IgM (1.2±0.35) was lower than that of the cases (3.5±2.5). Such information could be used to establish a baseline 'cut off' index value to differentiate symptomatic from asymptomatic cases following screening of a larger number of individuals.

Hantavirus infections can appear clinically uncharacteristic and may mimic other syndromes. This compounds the difficulties in diagnosing hantavirus infections in areas where the disease is not endemic and clinical cases may be sporadic[12]. Further, less pathogenic hantaviruses may cause a greater amount of asymptomatic infections, as seen for HFRS in Europe and Asia[13].

Though a good association was seen in the findings of the two serological assays, EIA and IFA, IFA was cumbersome and required specialized equipment and experienced personnel for accurate interpretation. The difference in positives between the two assays could be due to the fact that the EIA had a cocktail of baculovirus-derived recombinant nucleocapsid antigens from different hantavirus strains while in the IFA only Seoul virus antigen was used. The EIA offered the advantage of a rapid and less complicated detection system and could be done even in middle-level laboratories.

Positive serology should be interpreted very cautiously when done on single serum samples. Paired serum samples may help delineating true hantaviral infections. Further, the results of serology should be correlated with clinical features to arrive at a definitive diagnosis. Future studies need to be focused on confirmatory testing by detecting the hantaviral genome using RT-PCR in samples collected in the acute phase of illness.

Since no previous published data on hantavirus infections in India are available and not much is known about laboratory diagnosis or clinical cases, general physicians are probably unaware of diseases with hantavirus aetiology. This suggests that there could be circulation of the hantaviruses in the Indian population. Further studies need to be done to identify hantaviruses in clinical samples by molecular techniques to definitely describe the clinical picture of hantaviral infections in India. The molecular techniques will include primers that can identify Thottapalayam virus, the only hantavirus isolate from India. These techniques will also help to identify the rodent reservoirs and to elucidate the importance of potentially unknown hantaviruses in India.

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Identification and Characterization of an Immunodominant 28-Kilodalton *Coxiella burnetii* Outer Membrane Protein Specific to Isolates Associated with Acute Disease

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Coxiella burnetii **causes acute Q fever in humans and occasional chronic infections that typically manifest as endocarditis or hepatitis. Isolates associated with acute disease were found to be distinct from a group of chronic disease isolates by a variety of biochemical parameters and in a guinea pig fever model of acute disease, suggesting a difference in virulence potential. We compared antigenic polypeptides among *C. burnetii* isolates and found an immunodominant 28-kDa protein in acute group isolates but not in chronic group isolates (T. Ho, A. Hotta, G. Q. Zhang, S. V. Nguyen, M. Ogawa, T. Yamaguchi, H. Fukushi, and K. Hirai, Microbiol. Immunol. 42:81-85, 1998). In order to clone the *adaA* gene, the N-terminal amino acid sequence of *adaA* was determined and a 59-bp fragment was amplified from Nine Mile phase I DNA by PCR. The putative gene fragment was used to screen a lambda ZAP II genomic DNA library, and an open reading frame expressing a 28-kDa immunoreactive protein was identified. Sequence analysis predicted a gene encoding an ~28-kDa mature protein with a typical signal sequence. The *adaA* (acute disease antigen A) gene was detected in acute group *C. burnetii* isolates but not identified in chronic group isolates by PCR and Southern blotting. A typical signal peptide was predicted in *adaA*, and specific antibody to *adaA* reacted with the purified membrane fraction of acute group isolates by Western blotting, suggesting that *adaA* is exposed on the outer surface of *C. burnetii*. *adaA* was overexpressed in pET23a as a fusion protein in *Escherichia coli* to develop anti-recombinant *adaA* (anti-r*adaA*) specific antibody, which recognized a ~28-kDa band in acute group isolates but not in chronic group isolates. In addition, immunoblotting indicates that r*adaA* reacted with sera derived from animals infected with acute group isolates but did not react with sera from animals infected with chronic group isolates. These results support the idea that an *adaA* gene-targeted PCR assay and an r*adaA* antigen-based serodiagnostic test may be useful for differential diagnosis of acute and chronic Q fever.**

Coxiella burnetii is an obligate intracellular bacterium that causes acute and chronic forms of Q fever in humans. Acute Q fever is an influenza-like illness that usually is self-limiting and effectively treated by antibiotics (11). In contrast, chronic Q fever is a severe, sometimes fatal disease, and patients have responded poorly to various antibiotics (8, 20). Endocarditis is the most common chronic manifestation, while vascular infection, bone infection, and chronic hepatitis are also reported (21). Infection in most animals is mainly subclinical, but abortion and infertility are common manifestations in ruminants (2). Domestic animals, especially cattle, sheep, and goats, are important reservoirs of the agent responsible for infection of humans (7, 11).

C. burnetii has been isolated from various sources including milk, ticks, and humans with acute and chronic Q fever worldwide (2, 7, 8, 10). Previous studies have demonstrated that *C. burnetii* isolates originating from milk, ticks, and humans with acute Q fever differ in plasmid type (22), lipopolysaccharide profiles (3), and chromosomal DNA restriction endonuclease fragment patterns (5) from many isolates originating from chronic Q fever. The differences at the phenotypic and molecular levels between acute and chronic disease-associated isolates suggested that there may be a virulence potential characteristic of each group of isolates. Samuel et al. first reported that *C. burnetii* isolates associated with acute Q fever contained the QpH1 plasmid, while isolates associated with chronic Q fever possessed the QpRS plasmid or the plasmid sequences were integrated into the chromosome (22, 23). More recent studies of several *C. burnetii* isolates from Europe detected either the QpH1 plasmid-specific sequences (25, 26) or plasmid type QpDV (27) in both acute and chronic disease-associated isolates, suggesting that there was no specific gene(s) on plasmids responsible for a specific virulence phenotype. These data supported the notion that chronic disease could result from isolates associated with acute disease and might result from unique patient factors associated with immune status (25–27). However, no chronic disease-associated organisms have been isolated from acute Q fever patients. Therefore, it is quite possible that there are bacterial genetic factors responsible for acute disease. This hypothesis was supported in a

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TABLE 1. Original source, pathogenic characteristics, genetic group, and plasmid type of *C. burnetii* strains

Group[a]	Plasmid type[b]	Isolate[c]	Phase	Original source	Disease or type	Passage[d]
I	QpH1	Nine Mile RSA493	I	Montana, tick, 1935		307GP/1TC/1EP
		Turkey RSA333	II	Turkey, human blood, 1948	Acute	31EP
		African RSA334 (>[e])	I	Central Africa, human blood, 1949	Acute, Congolese red fever	3HP/4EP
		Giroud RSA431 (>[e])	I	Central Africa, human blood, 1949	Acute, Congolese red fever	2GP/2EP
		El Tayeb RSA342	I	Egypt, tick, 1967		4GP/2EP
		Panama RSA335	I	Panama, chiggers, 1961		4EP
		California 33 RSA329	I	California, cow's milk, 1947	Persistent	6EP
		Ohio 314 RSA270	I	Ohio, cow's milk, 1956	Persistent	4EP
II		Henzerling RSA331	II	Italy, human blood, 1945	Acute	36EP
IV	QpRS	Priscilla	I	Montana, goat cotyledon, 1980	Abortion	GP/2EP
		KQ154	I	Oregon, human heart valve, 1976	Endocarditis	HV/2EP
V	NP	GQ212	I	Nova Scotia, Canada, human heart valve, 1981	Endocarditis	HV/2EP
		SQ217	I	Montana, human liver biopsy sample, 1981	Hepatitis	BX/2EP
		KoQ229	I	Nova Scotia, Canada, human heart valve, 1982	Endocarditis	HV/2EP
VI	QpDG	Dugway 7E22-57	I	Utah, rodents, 1958		3EP
—[f]	QpDV	MAN	I	French, human blood	Aortic aneurysm	?
—[f]		ME	I	French, human heart valve	Endocarditis	?

[a] As defined by restriction enzyme banding patterns (5).
[b] Plasmids were described elsewhere (22, 27).
[c] Provided by Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Mont. Reference strains were determined by complement block titration (M. G. Peacock, Rocky Mountain Laboratories).
[d] Numbers indicate passage number; GP, guinea pig passage; TC, tissue culture; EP, egg passage; HP, hamster passage; HV, heart valve; BX, liver biopsy sample; ?, passage prior to receipt in authors' laboratory not known.
[e] >, passage history variants.
[f] —, MAN and ME were not classified (27).

study by Moos and Hackstadt (17) comparing virulence of a prototype isolate from each group in guinea pigs. The acute disease group prototype isolate (Nine Mile phase I RSA493) caused infection and fever when delivered intraperitoneally with less than 10 organisms, while the chronic disease group prototype isolate (Priscilla Q177) required at least 10^5 organisms to cause fever.

Our previous study identified a 28-kDa protein (P28) that was immunodominant in isolates originating from milk, ticks, and humans with acute Q fever but not immunogenic in isolates originating from chronic Q fever (6). This finding suggested that *adaA* could be associated with a pathogenic factor of acute Q fever. *adaA* may also have value as a marker to distinguish isolate groups. In order to clone and characterize the *adaA*-encoding gene, the N-terminal amino acid sequence of the protein was determined by protein sequencing. A 59-bp gene fragment was amplified from Nine Mile phase I DNA by PCR with one primer pair designed based on the N-terminal amino acid sequence and was used as a probe to screen a genomic library by Southern hybridization. The gene encoding P28 was cloned and sequenced. Outer membrane localization and antigenicity of *adaA* indicated that *adaA* may be a virulence factor related to acute Q fever, and the *adaA* gene may be a useful genetic marker for differentiation of isolates of *C. burnetii*.

MATERIALS AND METHODS

Bacterial strains, phage, and growth conditions. Seventeen *C. burnetii* isolates from various clinical and geographical sources were used in this study. The original source, pathogenic characteristics, and genetic properties of these strains are summarized in Table 1. All the isolates were propagated in BGM or L929 cell cultures and purified as described elsewhere (7, 22). The bacteriophage lambda ZAP II (Stratagene, La Jolla, Calif.) was used as the vector for construction of the *C. burnetii* expression genomic DNA library. *Escherichia coli* XL-Blue MRF' (Stratagene) was cultured in Luria broth (LB) with 12.5 μg of tetracycline/ml and used as the host strain for recombinant plasmids and bacteriophage lambda ZAP II.

Preparation of *C. burnetii* OMPs. The outer membrane proteins (OMPs) of *C. burnetii* were extracted from purified *C. burnetii* Nine Mile based on the method described by Ohashi et al. (19). Briefly, purified organisms were suspended in 10 mM sodium phosphate buffer, pH 7.4, containing 1% Sarkosyl (Sigma, St. Louis, Mo.) and 50 μg each of DNase I and RNase A and incubated at 37°C for 30 min. EDTA at a final concentration of 15 mM was added to stop the nuclease reaction. The insoluble precipitates were obtained by centrifugation at 10,000 × *g* for 1 h, washed twice with 0.1% Sarkosyl–phosphate-buffered saline, and then resuspended in STE buffer (100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride (Sigma).

Analysis of the N-terminal amino acid sequences of *adaA*. The OMPs of *C. burnetii* Nine Mile were separated by reversed discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a polyvinylidene difluoride membrane as described elsewhere (19). The presence of *adaA* in the purified membrane fraction of *C. burnetii* Nine Mile was confirmed by immunoblotting as described previously (6). The portion of the polyvinylidene difluoride membrane containing *adaA* was excised and analyzed with the HP G1005A protein sequencing system (Takara Shuzo Co., Kyoto, Japan).

Preparation of DNA probe specific to the P28-encoding gene. The N-terminal amino acid sequence of *adaA* was determined as ENRPILNTINYQQQVEKWV TTDSADVMVSVN. Based on the N-terminal amino acid sequence, a pair of primers, P28a (5'-ATHAAYTAYCARCARCARGT-3') and P28b (5'-AGCAT NACRTCNGC-3'), were designed and used to amplify a 59-bp fragment of the putative *adaA* gene. The expected 59-bp product was amplified from *C. burnetii* Nine Mile DNA by PCR with these primers. The nucleotide sequence of the 59-bp fragment was determined by the dideoxy nucleotide chain-termination method as described previously (29). Sequence analysis of the 59-bp fragment indicates that the deduced amino acid sequence is identical to the chemically determined N-terminal amino acid sequence of P28, suggesting that the 59-bp fragment is specific DNA of the *adaA* gene. Based on the determined nucleotide sequence, specific primers P28a1 (5'-ATTAATTATCAACAGCAGGTTG-3') and P28b1 (5'-AGCATTACATCGGCAGAATCC-3') were designed and used to amplify the 59-bp specific fragment of the *adaA* gene from *C. burnetii* Nine Mile DNA. The amplified 59-bp fragment was labeled by the random primer

extension method with the digoxigenin DNA labeling kit (Roche Diagnostics K. K., Tokyo, Japan) and used as a DNA probe to screen the genomic DNA library of *C. burnetii* by Southern hybridization.

Construction and screening of genomic DNA library. A lambda ZAP II genomic DNA library was constructed as described by Macellaro et al. (9) and screened by Southern hybridization with the *adaA* gene-specific probe. Briefly, the genomic library was plated on *E. coli* XL-Blue MRF′ to yield about 500 plaques per plate. Plates were incubated at 37°C until plaques were 1 mm in diameter. Plaques were transferred onto a nylon membrane (Amersham Pharmacia Biotech, Piscataway, N.J.) and were hybridized with the *adaA* gene-specific probe according to the protocol provided by the manufacturer (Roche Diagnostics K. K.). The positive plaques were detected by using the digoxigenin luminescent detection kit (Roche Diagnostics K. K.). In vivo excision of the pBluescript vector along with the inserted DNA of each positive clone was performed according to the protocol of the supplier of the lambda ZAP II cloning system.

Immunoblot analysis of *adaA* expression in *E. coli*. *E. coli* containing the recombinant plasmid was cultured in LB supplemented with 4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37°C overnight, and then cells were pelleted by centrifugation. The cell pellet was analyzed by SDS-PAGE and immunoblotting with rabbit anti-Nine Mile serum as described previously (30).

DNA sequence analysis. Plasmid DNAs from positive clones that expressed immunoreactive protein were isolated and purified by using the FlexyPrep kit (Amersham Pharmacia Biotech). The nucleotide sequence was partially determined by the dideoxy nucleotide chain-termination method with the Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit and SEQ4x4 personal sequencer system (Amersham Pharmacia Biotech). A BLAST search against the complete genomic sequence of Nine Mile phase I (24) was achieved to identify the complete nucleotide sequence of the cloned gene. The nucleotide sequence and the deduced amino acid sequence were analyzed by the GENETYX analyzing system (Software Development Co., Ltd., Tokyo, Japan).

Detection of the *adaA* gene from various isolates of *C. burnetii* by PCR. A pair of primers, P28F and P28R, was designed based on the *adaA* gene sequence and used to amplify a 269-bp fragment (ranging from positions 369 to 637 in the open reading frame [ORF] region of the *adaA* gene) from DNAs of 17 isolates from various clinical and geographical sources. The sequences of the primers are as follows: P28F, 5′-AATAGATTCGCTCTCTCAAGCCG-3′, and P28R, 5′-TCA CCGCTGTTTTTTCAGACG-3′. PCR was performed with 2.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, Calif.) in 50 μl of reaction mixture containing 20 ng of genomic DNA, 0.2 μM (each) primer, and 200 μM (each) deoxynucleotide triphosphates in 10 mM Tris-HCl (pH 8.3)–50 mM KCl–2.5 mM $MgCl_2$. The reactant was subjected to 35 cycles of 30 s at 94°C, 30 s at 53°C, and 1 min at 72°C in a DNA thermal cycler (PTC-0200 DNA Engine; MJ Research, Inc., Waltham, Mass.).

Southern blotting. Restriction enzyme-digested DNAs from various clinical phenotypic isolates including two acute prototypic isolates (Nine Mile and Henzerling) and five chronic prototypic isolates (Priscilla, Q217, Q229, MAN, and ME) were tested by Southern blotting with the *adaA* gene-specific probe.

Expression and purification of the *adaA* fusion protein. The 602-bp DNA fragment of the *adaA* gene was amplified from *C. burnetii* Nine Mile DNA by PCR with primers P28EF-P28ER, which were designed from the *adaA* gene sequence and included 602 bp of the ORF region without the signal peptide-encoding sequence. Primer P28EF (5′-TTCGCTGCCACCGGATCCTTC-3′) is the 5′ end of the *adaA* gene with an additional BamHI restriction site, and primer P28ER (5′-ATCAACTCGAGGTTTCTTCG-3′) is complementary to the 3′ end of the gene with a XhoI restriction site in the sequence. The amplified *adaA* gene fragment was digested with BamHI and XhoI, ligated to expression vector pET23a, and then transformed into *E. coli* BL21(DE3)LysS competent cells. Expression of T7-tagged (N-terminal) and His-tagged (C-terminal) recombinant *adaA* (r*adaA*) was induced by 4 mM IPTG. r*adaA* was purified by using a ProBond resin column (Invitrogen) under denaturing conditions.

Antiserum preparation and immunoblot analysis of *adaA* among various strains of *C. burnetii*. The anti-*adaA* specific antibody was produced by immunization of BALB/c mice with purified r*adaA*. Briefly, BALB/c mice (6 weeks old) were immunized with purified recombinant fusion protein in adjuvant (Titermax) three times at 14-day intervals. At each immunization, mice were subcutaneously injected with 50 μl of antigen (containing 20 μg of r*adaA*) mixture with 50 μl of Titermax. After the third immunization, serum was collected and stored at −20°C.

The expression of *adaA* in various strains of *C. burnetii* was confirmed by immunoblotting with anti-*adaA* specific serum. SDS-PAGE and immunoblotting were performed as described elsewhere (31).

Reactivity of purified r*adaA* with infection-derived sera. The reactivity of r*adaA* with sera from guinea pigs infected with various strains of *C. burnetii* was analyzed by immunoblotting. Guinea pig serum was collected at 4 weeks post-aerosol infection with 10^6 organisms of the Nine Mile phase I, Ohio, Q217, or Q229 strain and stored at −80°C until use. *C. burnetii* Nine Mile whole-cell lysate and purified rCom1, which is a protein common to all isolates tested (29, 30), were used as a control to confirm the presence of the antibodies to *C. burnetii* antigens in infection-derived sera. SDS-PAGE and immunoblotting were performed as described previously (31).

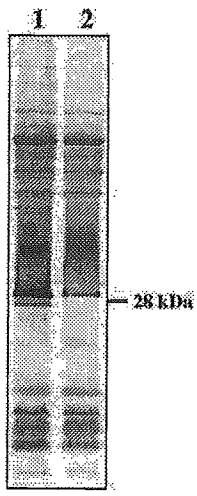

FIG. 1. Immunoblot analysis of purified OMPs of Nine Mile and Q217 strains with rabbit anti-Nine Mile hyperimmune serum. Lane 1, Nine Mile; lane 2, Q217.

RESULTS

Cloning the *adaA* gene. Immunoblotting identified an immunoreactive band at 28 kDa in the purified membrane fraction of *C. burnetii* Nine Mile but did not detect reactivity in the Q217 strain (Fig. 1). The result confirmed that the 28-kDa protein corresponds to the *adaA* previously noted (6). To identify the *adaA* gene, we determined the N-terminal 31 amino acids of a 28-kDa protein from *C. burnetii* Nine Mile. Based on the amino acid sequence, we designed several primer pairs and successfully amplified a 59-bp fragment from *C. burnetii* Nine Mile DNA by PCR. The 59-bp fragment was used as a DNA probe to screen a genomic library of *C. burnetii* Nine Mile DNA. Approximately 10,000 plaques were screened by Southern hybridization with the *adaA* gene-specific probe. Forty positive plaques were purified and compared for expression of immunoreactive proteins. Coomassie brilliant blue (CBB) staining on an SDS-polyacrylamide gel identified one clone, designated p110, expressed as an ~24-kDa protein. Immunoblotting indicates that the protein expressed by clone p110 reacted with rabbit anti-Nine Mile serum (data not shown), suggesting that the clone p110-expressed recombinant protein is specific for *C. burnetii*.

Sequence analysis. To determine the nucleotide sequence of the ORF encoding *adaA*, the purified recombinant plasmid from clone p110 was sequenced. The *adaA* gene-specific primers P28a1 and P28b1 were used in a sequence reaction to directly determine the nucleotide sequence of the *adaA* gene. The sequence of the p110 cloned insert was BLAST searched against the complete genome sequence of *C. burnetii* Nine Mile RSA493, allowing confirmation of the nucleotide sequence of

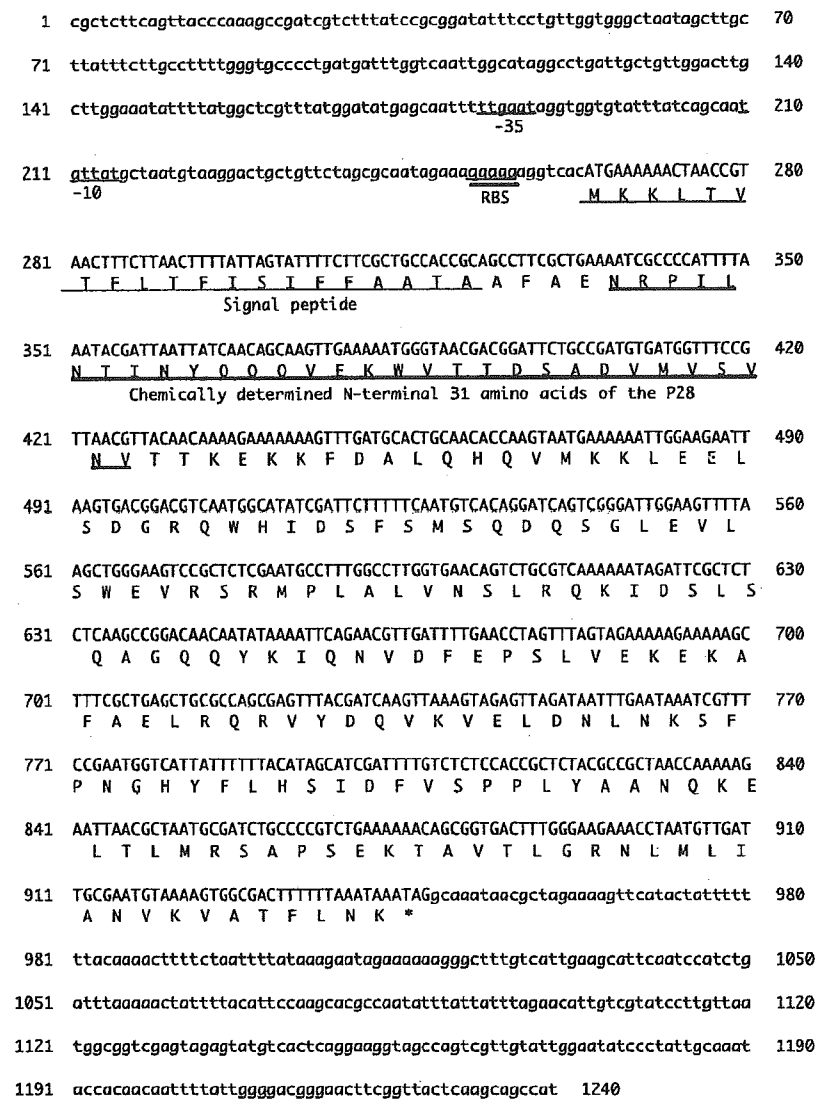

FIG. 2. Nucleotide sequence of the *adaA* gene. The deduced amino acid sequence is shown beneath the nucleotide sequence of the ORF. A putative promoter (positions −35 and −10) is underlined (thin line). The predicted ribosome-binding site (RBS) is underlined (thick line). The 20-amino-acid sequence at the N-terminal end of *adaA* is predicted to be a signal peptide. The chemically determined N-terminal 31-amino-acid sequence was identical to the N-terminal end of the deduced amino acid sequence of *adaA*.

the cloned gene (24). The nucleotide sequence (1,240 bp) from the cloned gene is shown in Fig. 2, which includes the flanking regions and the deduced amino acid sequence of P28. The *adaA* gene has a predicted ORF consisting of 684 bp, starting with an ATG codon at position 264 and ending with a TAG codon at position 947. The ORF is preceded by a putative ribosome-binding site, GGAGG, from 7 bp upstream of the ATG start codon. A predicted promoter sequence, TTGAA T-21 nt-TGTTAT, was found 36 bp upstream from the putative ribosome-binding site. The G+C content of the ORF coding region was 40%, which is similar to the value of *C. burnetii* total genomic G+C content (43%). The predicted mature P28 protein consists of 227 deduced amino acid residues and has a calculated molecular mass of 25,950 Da and a theoretical isoelectric point of 8.60. A 20-amino-acid signal peptide was also predicted for the N-terminal sequence of *adaA*. We also confirmed that the chemically determined N-terminal 31 amino acids of *adaA* were identical to the amino acid sequence of the mature protein deduced from the nucleotide sequence of the cloned *adaA* gene, which is the region immediately adjacent to a predicted signal peptide (Fig. 2). In addition, a BLAST search of GenBank with the deduced amino acid sequence of the *adaA* gene indicated that the ORF encoding *adaA* was identical to CBU0952, which was predicted by The Institute for Genomic Research gene annotation (24). These results suggest that the ORF identified in the p110 cloned gene sequence is a gene unique to *C. burnetii* and encodes *adaA*.

Detection of the *adaA* gene in various isolates of *C. burnetii*. PCR and Southern blotting were used to test and confirm

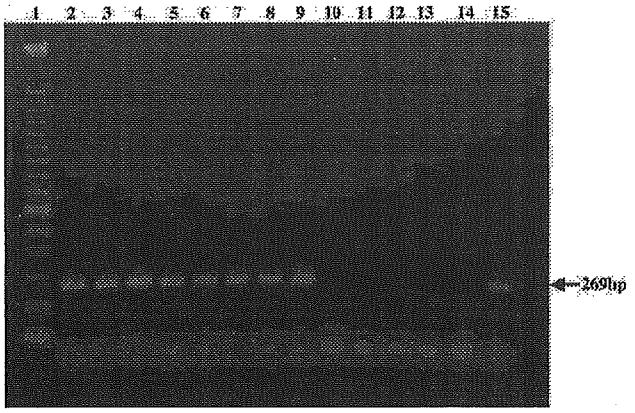

FIG. 3. Detection of the *adaA* gene from various isolates of *C. burnetii* by PCR with primers P28F-P28R. Shown is an ethidium bromide-stained agarose gel electrophoretogram of PCR-amplified products. Lane 1, molecular size markers (100-bp DNA ladder); lanes 2 to 9, isolates originating from ticks, milk, and humans with acute Q fever (Nine Mile, Ohio, California, El Tayeb, Africa, Panama, Turkey, and Giround, respectively); lanes 10 to 14, isolates originating from a goat and humans with chronic Q fever (Priscilla, KQ154, KoQ229, SQ217, and GQ212, respectively); lane 15, Dugway isolate.

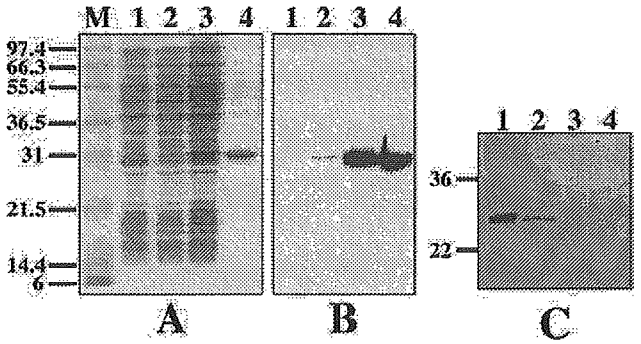

FIG. 4. Expression of the *adaA* gene in the pET23a expression vector system. Expression of the T7-tagged (N-terminal) and His-tagged (C-terminal) fusion protein was monitored by CBB staining of uninduced and induced cultures by SDS-PAGE and immunoblotting with His-tag-specific monoclonal antibody. (A) CBB staining profile of the expressed and purified fusion *adaA* protein. Lane M, molecular size markers; lane 1, pET23a-transformed *E. coli* (negative control); lanes 2 and 3, *adaA* gene recombinant pET23a-transformed *E. coli* uninduced and induced cultures, respectively; lane 4, purified fusion r*adaA*. (B) Immunoblot analysis of expressed and purified recombinant proteins with His-tag-specific monoclonal antibody. The samples shown in panel B are the same as in panel A. (C) Immunoblot analysis of *adaA* in various strains of *C. burnetii* with *adaA*-specific antiserum. Lanes 1 to 4, whole-cell lysate of Nine Mile, Henzerling, Priscilla, and Q217 strains, respectively. A ~28-kDa reaction band was observed in acute disease isolates including the Nine Mile and Henzerling strains but not detected in Priscilla and Q217. Numbers at left are molecular masses in kilodaltons.

whether the *adaA* gene is unique for a subgroup of isolates. The PCR result indicated that the *adaA* gene-specific fragment was amplified from isolates originating from humans with acute Q fever, ticks, cattle, and rodents, but PCR did not amplify any product from isolates from goats or humans with chronic Q fever (Fig. 3). Southern blotting also indicated that the *adaA* gene-specific probe hybridized with one band with SalI-digested DNAs of Nine Mile and Henzerling strains associated with acute Q fever but did not hybridize with any band with SalI-digested DNAs of Priscilla, Q217, Q229, MAN, and ME strains, which have been linked to chronic Q fever (data not shown). These results suggest that the *adaA* gene is specific for *C. burnetii* isolates originating from humans with acute Q fever, ticks, cattle, and rodents.

Expression of *adaA* in *E. coli* and various isolates of *C. burnetii*. The partial *adaA* protein of the Nine Mile strain was overexpressed as a fusion protein in pET23a. An IPTG-inducible fusion protein with a molecular mass of 28 kDa was detected in the *adaA* gene recombinant pET23a-transformed *E. coli* culture by CBB staining of the SDS-polyacrylamide gel and immunoblotting with a His-tagged specific monoclonal antibody (Fig. 4A and B, lanes 2 and 3). The expressed fusion protein was not detected in the negative control of pET23a-transformed *E. coli* culture (Fig. 4A and B, lanes 1). SDS-PAGE and immunoblotting also indicated that r*adaA* was successfully purified from the *adaA* gene recombinant pET23a-transformed *E. coli* culture by using ProBond resin column (Fig. 4A and B, lanes 4). To confirm that P28 is expressed by acute disease isolates but not carried by chronic disease isolates, anti-r*adaA* specific antibody was produced and used in immunoblotting with antigens of various strains of *C. burnetii*. Immunoblotting indicated that a ~28-kDa reaction band was detected from acute-disease-associated isolates Nine Mile and Henzerling but not observed in chronic-disease-associated isolates Priscilla and Q217 (Fig. 4C). This result confirmed that our cloned *adaA* gene encodes *adaA* and that *adaA* is expressed by acute-disease-associated isolates but not carried by chronic-disease-associated isolates.

Reactivity of purified r*adaA* with sera derived from infected animals. Figure 5 shows the immunoblots of the whole-cell antigen, rCom1, and r*adaA* with sera derived from a guinea pig

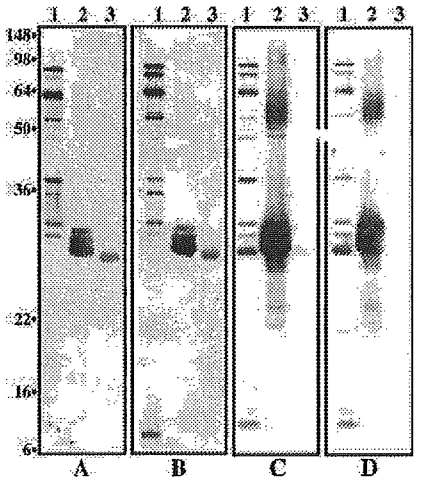

FIG. 5. Immunoblots of whole-cell antigen, rCom1, and r*adaA* with sera derived from guinea pigs 4 weeks post-aerosol infection with 10^6 organisms of either the acute or the chronic prototypic isolate of *C. burnetii*. Sera (diluted 1:500) came from guinea pigs infected with Nine Mile (A), Ohio (B), Q217 (C), and Q229 (D). The samples shown in panels A to D are the same. Lanes 1, Nine Mile whole-cell antigen; lanes 2, purified rCom1 protein; lanes 3, purified recombinant *adaA* protein. Numbers at left are molecular masses in kilodaltons.

4 weeks post-aerosol infection with 10^6 organisms of the acute or chronic prototypic strain of *C. burnetii*. All sera from guinea pigs aerosol infected with 10^6 organisms of various strains strongly reacted with Nine Mile whole-cell antigen at a wide range of molecular weights and with rCom1 at similar levels (Fig. 5, lanes 1 and 2). However, r*adaA* reacted with sera from guinea pigs infected with isolates Nine Mile and Ohio (Fig. 5A and B, lanes 3) but not with sera from those infected with isolates Q217 and Q229 (Fig. 5C and D, lanes 3). These results indicate that anti-*adaA* specific antibody was present in sera derived from animals infected with the acute prototypic isolate but absent in sera from animals infected with the chronic prototypic isolate.

DISCUSSION

Cloning and characterization of *adaA* demonstrated that this protein is specific for acute-Q-fever-related isolates but deleted in chronic-disease-associated isolates despite geographical source, suggesting that *adaA* may be a virulence factor involved in the pathogenesis of acute Q fever in humans.

The predicted *adaA* mature protein consists of 227 amino acids and has a predicted molecular mass of 25,950 Da. This is very close to the molecular size of native *adaA* expressed in *C. burnetii* but about 2 kDa larger than the expression product of the *adaA* gene in *E. coli* (data not shown). The 25-amino-acid signal peptide is predicted in the N-terminal sequence of *adaA*, which is probably cleaved from the mature protein when the *adaA* gene is expressed in *E. coli*. The chemically determined N-terminal and internal peptide (data not show) amino acid sequences of *adaA* were identical to the deduced amino acid sequence of the cloned *adaA* gene, confirming that the identified ORF encodes *adaA*. The cloned *adaA* gene recombinant pUC19 expressed r*adaA* in *E. coli* DH5α cells without induction by IPTG (data not shown). A potential promoter sequence, TTGAAT-21 nt-TGTTAT, was identified in the *adaA* gene sequence, suggesting that the *adaA* gene was expressed in *E. coli* by using the endogenous promoter. A BLAST search of GenBank with either the nucleotide sequence or the deduced amino acid sequence for the *adaA* gene did not identify significant DNA or amino acid homologies, suggesting that *adaA* is unique to *C. burnetii*.

OMPs of gram-negative bacteria are employed in several important roles in the host-parasite interaction and relate to both pathogenesis and protective immunity. Due to the difficulties in cultivation and purification of *C. burnetii*, only a limited group of OMP-encoding genes have been characterized (4, 16, 28). Candidates for OMPs include the QpH1 plasmid-specific gene *cbhE'* for a 42-kDa surface protein (15) and the QpRS plasmid-specific gene *cbbE'* for a 55-kDa surface protein (12–14), which have been speculated to be virulence related and associated with acute or chronic Q fever in humans. However, recent investigations of several European isolates suggested that there were no specific genes on plasmids responsible for acute or chronic Q fever (25–27) and supported the notion that host factors may play a key role in the development of chronic Q fever. It remains unknown whether there are specific genes on the chromosome responsible for acute or chronic Q fever. Isolates from acute disease are distinct from chronic-disease-associated isolates at the molecular level (3, 5, 22) and in a guinea pig fever model of acute disease (17; K. Russell, unpublished data), suggesting different virulence potentials for groups of isolates of *C. burnetii*. In this study, we identified a novel ~28-kDa membrane-associated protein and demonstrated that *adaA* is expressed in acute group isolates but not carried by chronic group isolates, suggesting that *adaA* may be a virulence factor related to acute Q fever. Immunoblotting with purified r*adaA* antigen recognized anti-*adaA* specific antibody from sera derived from animals infected with acute group isolates but not from sera from animals infected with chronic group isolates, suggesting that *adaA* is an important antigen in acute disease. Since there has been no suitable animal model developed to represent the manifestation of chronic Q fever and because there is a lack of genetic tools for *C. burnetii*, it is not possible to directly test whether a specific gene is related to acute or chronic disease. Recently, SCID mice have been used as a model highly sensitive to lethal challenge by an acute-disease-associated isolate of *C. burnetii* (1), and preliminary comparison in this model shows dramatic differences in disease from isolates which do not carry *adaA* (M. Andoh, unpublished data). Further studies to test whether the *adaA* gene can be delivered on a stable plasmid to a *adaA*-negative isolate may allow its role in virulence to be determined.

Since prompt antibiotic therapy could lead to a better prognosis for individual patients with chronic Q fever, developing a diagnostic method for rapid differential diagnosis of acute and chronic Q fever could be very important for control of chronic disease. Recently, based on point mutations unique to isolate groups, *com1* and *icd* genes have been used as genetic markers to distinguish acute and chronic isolates (18, 29). However, comparison of nucleotide sequences of *com1* and *icd* genes among isolates indicates that they are highly conserved between acute and chronic isolates, except for these few point mutations (18, 29). The finding that the *adaA* protein and the *adaA* gene are unique to acute group isolates can be used for development of r*adaA* antigen-based serodiagnostic methods and/or an *adaA* gene-targeted PCR assay for differential diagnosis of acute and chronic Q fever in clinical samples. We have designed primers based on the nucleotide sequence of the *adaA* gene and used them to amplify products from DNA of various strains of *C. burnetii*. Amplicon products were amplified from DNA templates of isolates originating from humans with acute Q fever, ticks, cattle, and rodents but not from isolates originating from humans with chronic Q fever, suggesting that PCR for the *adaA* gene can be used for differentiation of acute- and chronic-disease-associated isolates. In addition, immunoblotting indicated that r*adaA* reacted with sera derived from animals infected with acute group isolates but was not recognized by sera derived from animals infected with chronic group isolates, suggesting that an r*adaA* antigen-based serodiagnostic test may be useful for differential diagnosis of acute and chronic Q fever in human sera. Further studies will evaluate the usefulness of an *adaA* gene-targeted PCR assay and an r*adaA* antigen-based enzyme-linked immunosorbent assay for differential diagnosis of acute and chronic Q fever in clinical samples from acute and chronic Q fever patients.

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Yersinia enterocolitica Serovar O:8 Infection in Breeding Monkeys in Japan

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***Abstract*: In the period from December 2002 to January 2003, 5 of 50 squirrel monkeys (*Saimiri sciureus*) housed at a Zoological Garden in the Kanto region of Japan died following a few days' history of diarrhea. After this outbreak had ended in the squirrel monkeys, 1 of 2 dark-handed gibbons (*Hylobates agilis*) died in April of 2003, showing similar clinical signs. We examined the organs of 3 of the dead squirrel monkeys and of the dark-handed gibbon, and *Yersinia enterocolitica* serovar O:8, which is the most pathogenic serovar of *Y. enterocolitica*, was isolated. In order to determine the source and the transmission route of infection, 98 fecal samples (45 from squirrel monkeys, 20 from other monkeys of 18 different species, and 33 from black rats captured around the monkey houses) and 7 water samples were collected in the Zoological Garden, and were examined for the prevalence of *Y. enterocolitica* serovar O:8. Serovar O:8 was isolated from 21 of 65 monkeys (32.3%) and 5 of 33 (15.2%) black rats (*Rattus rattus*). Furthermore, we examined the 30 isolates using molecular typing methods, pulsed field gel electrophoresis (PFGE), ribotyping using the RiboPrinter system, and restriction endonuclease analysis of virulence plasmid DNA (REAP), and compared the isolates in this outbreak with Japanese O:8 isolates previously identified. Genotyping showed that almost all the isolates were identical, and the genotype of the isolates was highly similar to that from wild rodents captured in Niigata Prefecture. This is the first report of fatal cases of *Y. enterocolitica* serovar O:8 infection in monkeys anywhere in the world.**

***Key words*: *Yersinia enterocolitica* serovar O:8, Pulsed field gel electrophoresis (PFGE), Ribotyping, Breeding monkey**

The term yersiniosis refers to infections caused by either *Yersinia enterocolitica* or *Yersinia pseudotuberculosis*, which appear as enteritis and sometimes septicemia in humans and animals (17, 26). Monkey species, especially New World monkeys such as the squirrel monkey (*Saimiri sciureus*), seem to be sensitive to *Y. pseudotuberculosis*. Many cases of yersiniosis in breeding monkeys have been reported, and *Y. pseudotuberculosis* in particular frequently causes fatal infection (6, 15, 21, 25, 27, 29, 32, 34, 37, 39). There have also been some reports of monkey infection with pathogenic *Y. enterocolitica* (3, 7, 28, 31, 36); however, no such infection has yet been reported in Japan. We report here on an outbreak of *Y. enterocolitica* serovar O:8, the most pathogenic serovar of this bacterium (4, 12, 23), in breeding monkeys at a Zoological Garden in the Kanto region of Japan, which we observed in the process of investigating occurrences of the *Yersinia* infection in breeding monkeys.

Materials and Methods

Case history. Between December 2002 and January

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Abbreviations: CFU, colony forming unit; IN, irgasan-novobiocin; LB, Luria-Bertani, NT, not tested; PBS, phosphate-buffered saline; PFGE, pulsed field gel electrophoresis; REAP, restriction endonuclease analysis of virulence plasmid DNA; TSA, trypticase soy agar; *Y. enterocolitica*, *Yersinia enterocolitica*; *Y. pseudotuberculosis*, *Yersinia pseudotuberculosis*.

2003, 5 of 50 squirrel monkeys housed at a Zoological Garden in the Kanto region located in central Honshu island, Japan, died following a few days' history of diarrhea. This outbreak was ended by treatment with antibiotics; however, despite this treatment, 1 of 2 dark-handed gibbons (*Hylobates agilis*) also died in April 2003, showing similar clinical signs. All of the dead monkeys showed severe enteritis, swelling of Peyer's patch and multiple abscesses in the spleen and liver.

This Zoological Garden keeps many species of monkeys. Squirrel monkeys are housed in an indoor-outdoor enclosure located on the northern edge of the Garden, and many small wild animals, such as small rodents, have easy access to the outdoor area. Additionally, the other monkeys are housed in outdoor cages which are about 50 m from the squirrel monkey enclosure.

Specimens. After 3 of the squirrel monkeys and the dark-handed gibbon died, their bodies were immediately transported to the laboratory, where they were dissected and their organs aseptically removed. Fecal samples were collected from 45 squirrel monkeys, from 20 other monkeys of 18 different species and from 33 black rats (*Rattus rattus*) captured around the monkey houses. Water samples were also collected at 7 points, drains and pools, in the monkey houses. All samples were immediately transported to the laboratory under cool conditions and examined for the presence of *Yersinia* spp.

Isolation and identification of Yersinia *spp.* The organs (liver, spleen, lung, small intestine, and intestinal content) of the dead monkeys were homogenized in phosphate-buffered saline (PBS; pH 7.2) and 10-fold serial dilutions of the suspension were plated on irgasan-novobiocin (IN) agar plates (10). All PBS suspensions were incubated at 4 C for 3 weeks as a cold enrichment. Thereafter, the suspensions were submitted to alkali treatment by mixing 0.1 ml of the suspensions with 0.9 ml of 0.4% KOH for 20 sec, and plated on IN agar (2). Feces (about 1 g) were suspended in 9 ml of PBS, and the PBS suspensions were treated as described above. Water samples were centrifuged for 15 min at 8,000 rpm, and the sediments were resuspended in PBS for obtaining 200-fold concentrations of the components of the original suspension, following the method of Fukushima (9). These concentrations were then plated on an IN agar plate after alkali treatment.

The plates were incubated at 25 C for 48 hr. Colonies morphologically similar to those of *Yersinia* spp. were subcultured with trypticase soy agar (TSA; BBL, Sparks, Md., U.S.A.) for biochemical examination. In brief, biochemical characteristics were examined on triple sugar iron medium (Eiken Chemical Co., Ltd., Tokyo), lysine indole motility medium (Nissui Pharmaceutical Co., Ltd., Tokyo), and urea broth (Eiken). If the following typical reactions of *Yersinia* spp., glucose and urease positive, gas and lysine decarboxylase and H_2S negative, were seen, additional biochemical tests were performed with the methods of Wauters et al. (40). Serotyping of *Y. enterocolitica* was accomplished by slide agglutination with a commercial rabbit anti-*Y. enterocolitica* sera set (Denka-Seiken, Co., Tokyo). To evaluate potential pathogenicity, *Y. enterocolitica* serovar O:8 isolates were examined for temperature-dependent calcium requirement by the method of Gemski et al. (11) and for temperature-dependent autoagglutination by the method of Laird and Cavanaugh (24).

Pulsed field gel electrophoresis (PFGE). Chromosomal DNAs from strains cultured overnight at 25 C in 10 ml of Luria-Bertani (LB) broth (Difco, Detroit, Mich., U.S.A.) were prepared using a CHEF Bacterial Genomic DNA Plug Kit (Bio-Rad Laboratories, Inc., Hercules, Calif., U.S.A.) according to the manufacturer's instructions. The DNAs were digested by the restriction enzyme *Not*I (TaKaRa, Shiga, Japan) in the reaction mixture. The DNA fragments were separated in 1.2% agarose NA (Amersham Pharmacia Biotech, Uppsala, Sweden) that was prepared in 0.5× Tris-borate-EDTA buffer (50 mM Tris base, 50 mM boric acid, 2 mM EDTA) on a CHEF-DRIII Pulsed Field Electrophoresis System (Bio-Rad). Electrophoresis was carried out for 24 hr at 14 C and 200 V with pulse times of 1 to 25 sec following the method of Buchrieser et al. (5). A CHEF DNA Size Standard Lambda Ladder (Bio-Rad) was used as a DNA size marker. The gels were stained with ethidium bromide for 2 hr, destained in distilled water, and photographed under UV light. Relatedness among PFGE patterns was analyzed based on the guidelines described by Tenover et al. (38). Criteria for interpreting PFGE patterns described by Tenover et al. is as follows. Isolates might be considered identical when PFGE patterns contain the same number and sizes of fragments; closely related when they differ by one to three bands; possibly related when they differ by four to six bands; and different when they differ by seven or more bands.

Ribotyping. Ribotyping was performed using the RiboPrinter System (Qualicon, Inc., Wilmington, Del., U.S.A.), and proprietary reagents (Qualicon), according to the manufacturer's instructions. Bacterial strains were incubated at 25 C for 48 hr on TSA and suspended in 200 µl of sample buffer. Thirty microliters of the suspension were transferred to the sample carrier and heated at 80 C for 10 min. After adding lytic enzymes and loading the bacterial cells and all the consumables into the system, the bacterial cells were automatically lysed, and the released DNA was digested with a restriction endonuclease *Eco*RI. The DNA restriction

fragments were size-separated by electrophoresis on an agarose gel, transferred to a nylon membrane, denaturated and hybridized with a labeled rRNA operon probe. After the addition of a chemiluminescent substrate, the light intensity of the obtained targeted DNA fragments composing DNA fragment patterns, namely ribopatterns, were captured by a customized CCD camera, converted to digital information, and stored in the system's computer data base. The ribopatterns with a similarity coefficient higher than 0.93 were considered identical by the RiboPrinter software and were grouped together in a same ribotype.

Restriction endonuclease analysis of plasmid DNA (REAP). Plasmid DNA was prepared following the method described by Kado and Liu (20) with some modifications. Briefly, REAP was performed with the enzymes *Eco*RI (TaKaRa) and *Bam*HI (TaKaRa) as described by Nesbakken et al. (30). Electrophoresis was performed for 105 min at 50 V in a 1.2% agarose NA gel using a Mupid-2 (Advance Co., Tokyo). A 1 kb PLUS DNA ladder (Invitrogen Co., Carlsbad, Calif., U.S.A.) was used as a DNA size marker. The gels were stained with ethidium bromide for 10 min, and photographed under UV light.

Table 1. Isolation of *Yersinia enterocolitica* serovar O:8 from organs of dead monkeys

No.	Monkey species	The number of bacteria in organs (log CFU/g)					
		Liver	Spleen	Lung	Small intestine	Intestinal content	Mandibular abscess [c]
1	Common squirrel monkey (*Saimiri sciureus*)	6.7	7.8	6.7	8.6	7.7	NT[b]
2	Common squirrel monkey (*Saimiri sciureus*)	+[a]	+[a]	3.9	2.5	+[a]	NT[b]
3	Common squirrel monkey (*Saimiri sciureus*)	NT[b]	NT[b]	NT[b]	NT[b]	NT[b]	+[a]
4	Dark-handed gibbon (*Hylobates agilis*)	8.0	7.9	6.1	NT[b]	6.3	NT[b]

[a] Detected after cold enrichment (the case of mandibular abscess, detected with smear culture).
[b] Not tested.
[c] Smeared culture.

Table 2. Isolation of *Yersinia enterocolitica* serovar O:8 from breeding monkeys and environmental materials in the Zoological Garden

	Source	Number of animals examined	Number of serovar O:8 isolates (%)
Breeding monkeys	Common squirrel monkey (*Saimiri sciureus*)	45	17 (37.8)
	Common chimpanzee (*Pan troglodytes*)	2	1 (50.0)
	Crab-eating macaque (*Macaca fascicularis*)	1	1 (100.0)
	De Brazza's monkey (*Cercopithecus neglectus*)	1	1 (100.0)
	Vervet monkey (*Cercopithecus aethiops*)	1	1 (100.0)
	Geoffroy's spider monkey (*Ateles geoffroyi*)	2	0 (0.0)
	Abyssinian colobus (*Colobus guereza*)	1	0 (0.0)
	Black-capped capuchin (*Cebus apella*)	1	0 (0.0)
	Black spider monkey (*Ateles paniscus*)	1	0 (0.0)
	Dark-handed gibbon (*Hylobates agilis*)	1	0 (0.0)
	Hamadryas baboon (*Papio hamadryas*)	1	0 (0.0)
	Japanese macaque (*Macaca fuscata*)	1	0 (0.0)
	Lesser spot-nosed monkey (*Cercopithecus petaurista*)	1	0 (0.0)
	Lion-tailed macaque (*Macaca silenus*)	1	0 (0.0)
	Mandrill (*Mandrillus sphinx*)	1	0 (0.0)
	Patas monkey (*Erythrocebus patas*)	1	0 (0.0)
	Ruffed lemur (*Varecia variegata*)	1	0 (0.0)
	White-handed gibbon (*Hylobates lar*)	1	0 (0.0)
	White-throated capuchin (*Cebus capucinus*)	1	0 (0.0)
	subtotal	65	21 (32.3)
Environmental materials	Black rat (*Rattus rattus*)	33	5 (15.2)
	Water	7	0 (0.0)
	subtotal	40	5 (12.5)
	Total	83	26 (31.3)

Results

Isolation of Y. enterocolitica *Serovar O:8 from Monkeys and Black Rats*

The numbers of *Y. enterocolitica* serovar O:8 (log CFU/g) isolated from each organ are summarized in Table 1. This pathogen was isolated from all tested organs of the dead monkeys. From No. 1 and 4 especially, high numbers of viable bacteria (log CFU/g) were isolated from livers (6.7 and 8.0), spleens (7.8 and 7.9), lungs (6.7 and 6.1), small intestine (8.6 in No. 1) and intestinal contents (7.7 and 6.3). From the other fecal samples examined, serovar O:8 was isolated from 21 of 65 monkeys (32.3%; 17 squirrel monkeys, 1 chimpanzee, 1 velvet monkey, 1 De Brazza's monkey, and 1 crab-eating macaque) and 5 of 33 (15.2%) black rats, but it was not isolated from any water samples (Table 2).

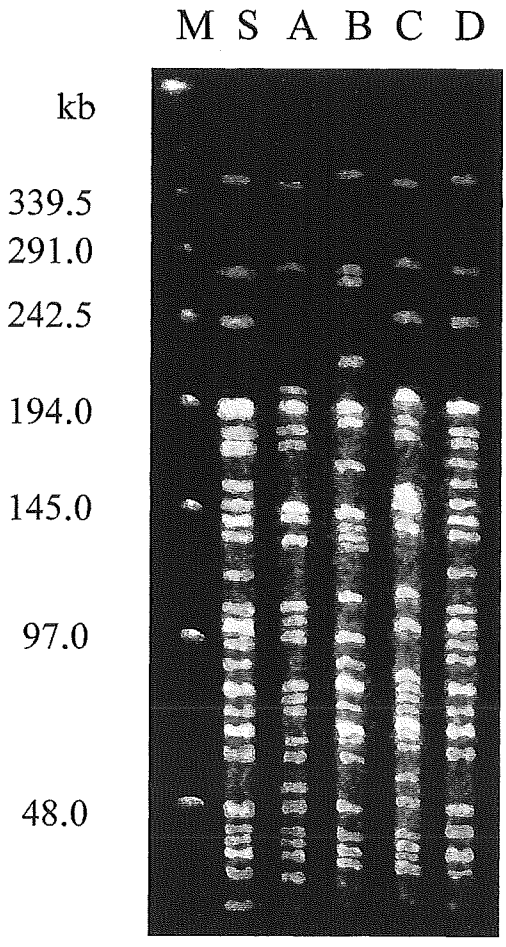

Fig. 1. Comparison of the PFGE patterns of representative outbreak isolate (lane S) and Japanese isolates (lanes A to D). Lanes: M, molecular weight marker; S, NY0212001 (squirrel monkey); A, YE89023 (human patient); B, NY936005 (wild rodent); C, NY9504002 (wild rodent); D, YE9809001 (wild rodent).

All isolates showed a positive reaction for virulence-associated properties such as calcium dependency and autoagglutination, and harbored a 70-kb virulence plasmid (data not shown). These isolates were therefore identified as pathogenic serovar O:8 strains.

Molecular Genotyping of Y. enterocolitica *Serovar O:8 Isolates*

The 30 isolates, consisting of 4 isolates from the organs of the dead monkeys (1 from each monkey), and 26 isolates from the other samples, were analyzed using molecular genotyping methods and showed the same PFGE pattern S (Fig. 1). Japanese O:8 isolates were previously identified and classified into four patterns designated as A to D (14). Compared with those patterns, our pattern S was almost identical to pattern D, differing by only two bands; according to the proposal made by Tenover et al. (38), these strains can thus be considered closely related.

The same strains were grouped into two ribotypes by the RiboPrinter (Fig. 2): 29 isolates were identified as S1, and 1 isolate from a black rat was identified as S2. Comparing these two ribotypes with the four ribotypes (R1 to R4) obtained from the same Japanese isolates submitted to PFGE, the ribotype S1 strains were assigned to the same R1 ribotype group, while S2 was assigned to ribotype R2 (14).

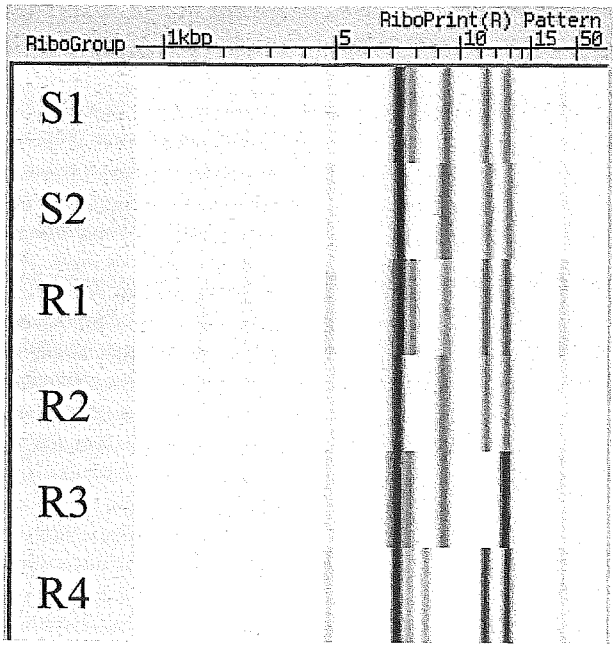

Fig. 2. Comparison of the ribotypes of representative outbreak isolates (lanes S1 and S2) and Japanese isolates (lanes R1 to R4). Lanes: S1, NY0212001 (squirrel monkey); S2, NY0304008 (black rat); R1, NY936005 (wild rodent); R2, YE9809001 (wild rodent); R3, YE87069 (human patient); R4, YE89023 (human patient).