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MOLECULAR EPIDEMIOLOGICAL ANALYSIS OF BARTONELLA ISOLATES FROM THE USA, FRANCE, AND JAPAN BY PULSED FIELD GEL ELECTROPHORESIS

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Des travaux récents ont montré que l'agent causal de la maladie des griffes du chat (CSD) est un bacille polymorphe gram négatif, Bartonella henselae. Pourtant, il reste très difficile d'examiner les caractéristiques biochimiques et épidémiologiques des isolats car la croissance de ce micro-organisme est fastidieuse et ses biotypage et ribotypage sont encore mal connus. En bactériologie, des électrophorèses sur gel en champ pulsé (PFGE) sont utilisées pour déterminer la taille, la carte de l'ADN bactérien chromosomal et pour faire l'analyse épidémiologique de certaines maladies. Cette étude a pour objectif de rassembler des informations sur l'épidémiologie de B. henselae isolé aux U.S.A., en France et Japon par PFGE (CHER-DR III, BIO-RAD).
Quatre enzymes de restriction (Note1, Asc1, Sma1, Eag1) ont été sélectionnées pour analyser l'ADN de Bartonella.
Bien que la plupart des isolats aient montré différents profils d'électrophorèse, quelques isolats avaient des profils identiques. Deux profils différents d'ADN ont été observés à partir de souches françaises et japonaises isolées chez les mêmes chats, indiquant une co-infection. La taille de l'ADN chromosomal de B. henselae variait de 1.6 à 2.4 Mb. Les ADN qui n'ont pas été coupés par Not1 ont été analysés par Asc1, Sma1 et Eag1. Certains isolats de différents pays ont des profils identiques d'ADN. Bartonella clarridgeiae (nouvelle espèce) a montré un profil spécifique à partir de Sma1 et Asc1 et l'infection a été trouvée chez des chats provenant des USA, de France mais pas du Japon.
Dans cette étude, les faits suivants ont été observés : 1) la taille de l'ADN du génome de B. henselae a été estimée entre 1.6 et 2.4 Mb. 2) les électrophorèses (PFGE) utilisant Not1, Asc1,Sma1 et Eag1 constituent un outil intéressant pour la différentiation des souches de B. henselae. 3) la taille du génome de B. clarridgeiae était d'environ 1.6 Mb et l'infection des chats semble prévalente aux USA et en France mais pas au Japon. 4) Certains chats ont été co- et/ou ré-infectés avec différentes souches et/ou espèces. 5) Plusieurs isolats des différents pays ont les mêmes profils d'ADN chromosomal.

The causative agent of cat scratch disease (CSD) has remained unknown for a long time. Recent investigations have suggested that *Bartonella henselae*, formerly named *Rochalimaea henselae* is a causative agent for bacillary angiomatosis and bacillary peliosis in HIV positive patients [10]. Furthermore, the organism was isolated from the CSD patients [2] and serological investigations on the patients strongly suggested that the organism was the agent of CSD [8,12].
It is very difficult to examine the biochemical characteristics of *B. henselae* because of its fastidious property. An unique method for the identification of the bacteria is polymerase chain reaction(PCR) and restriction enzyme cutting fragment analysis of PCR products [9]. However, this method can not provide the differentiation of the strains. Furthermore, biotyping and serotyping of the organism have not been established so far.
Recently, pulsed field gel electrophoresis (PFGE) has been developed. It has been possible to analyze large size of DNA. In the field of bacteriology, PFGE is used for sizing and mapping of bacterial chromosome DNA [11]. Furthermore, PFGE is applied to the epidemiological analysis of some infectious diseases [3,5,6]. Though intra- and interspecies differentiation of *B. henselae* by PFGE have been reported [7], there is no reports to compare the detailed chromosomal DNA profiles of the strains from different countries.
The present study is conducted to differentiate *B. henselae* isolated in the USA, France, and Japan by PFGE and to get some information in the epidemiology of CSD.

MATERIALS AND METHODS

A total of 29 *B. henselae* isolated from cats in the USA (8 strains), France (8 strains), and Japan (13 strains) were analyzed. The strains were cultured on 5% rabbit blood agar plates at 35°C for 7 ~14 days under 5% CO2 atmosphere. The bacteria grown on the agar plates were scraped and suspend in sterile distilled water and washed twice with sterile water by centrifugation at 12,000 rpm for 5min. The turbidity of the suspension was adjusted at McFarland # 6. The suspension was mixed gently but thoroughly with same amount of 2% agarose. The mixture was solidified in plug molds at room temperature. The agarose plugs were incubated in lysozyme solution (1mg/ml) at 37°C overnight. Then, the plugs were rinsed with sterile water and incubated with protenase K solution (1mg/ml) at 50°C over night without agitation. New protenase K solution was replaced and incubated with same conditions. The plugs were washed 4 times in 10 ml of wash buffer for 1 hr at room temperature with gently agitation. Protenase K was inactivated by 1mM phenyl methyl sulfonyl fluoride during second or third wash.

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Four restriction enzymes (Not1, Asc1, Sma1, Eag1) were used for the study. The plugs were incubated in appropriate conditions following the instruction manuals of the enzymes.
Electrophoresis conditions were followed by the Table I.

Table I
The PFGE condition for each enzyme

Enzyme	Recognition site	Voltage	Pulse time	Run time	Agarose concentration
Not1	GC ↓ GGCCGC	4.5V/cm	5-120S	33hr	1.0% agarose
Asc1	GG ↓ CGCGCC	4.5V/cm	5-120S	33hr	1.0% agarose
Sma1	CCC ↓ GGG	5.7V/cm	3- 10S	26hr	1.5% agarose
Eag1	C ↓ GGCCG	5.7V/cm	2- 7S	26hr	1.5% agarose

RESULTS

In Not1 digestion, 7 of 8 American strains were digested to 5 to 8 fragments. Most strains showed specific fragment profiles but 2 strains from different cats showed almost same profiles. Six of 8 French strains were cut to 5 to 7 fragments. The chromosomal DNA of 1 American and 2 French strains were not digested by Not1. Of 13 Japanese strains from four cats, although most strains from the same cats showed the same digestion profiles, 3 strains showed different DNA profiles in comparison with other strains isolated from the same cats.
In Asc1 digestion, the strain which wasn't cut by Not1 was cut to 5 fragments. They showed all same digestion profiles.
In Sma1 digestion, all strains from different countries could be clearly differentiated by the enzyme. Though 2 French and 1 American strains which were not cut by Not1, they were clearly differentiated by Sma1 digestion and the profiles of the strains were quite different from other strains. They were identified as new bartonella species *B. clarridgeiae* by DNA sequencing. The difference of Sma1 digestion profiles of Japanese strains coincides with the difference of Not1 digestion profiles.
In Eag1 digestion, the profiles of all strains coincided with the difference of digestion profiles of other enzymes.

DISCUSSION

The results of Not1 and Asc1 digestion showed that genome DNA size of *B. henselae* was estimated between 1.6 Mb and 2.4 Mb. This result was almost same as the report of Roux et al [7].
Some Japanese and French strains isolated from the same cats showed different digestion profiles in comparison with other strains. The data suggest that some cats harbored plural strains or were reinfected with different strains of *B. henselae*.
Recently, the isolation of an unique Bartonella-like strain from the cat of a HIV-positive patient from which *B. henselae* was isolated was reported by Clarridge et al [1]. The name *Bartonella clarridgeiae* sp. nov. is proposed for this novel bacterium [4]. In Asc1 and Sma1 digestion, all *B. clarridgeiae* showed same digestion profiles and they were found among American and French strains but not in Japanese strains. These data suggest that the genome size of *B. clarridgeiae* is about 1.6 Mb and Asc1 and Sma1 are useful enzymes to differentiate B. clarridgeiae from *B. henselae*. Furthermore, it seemed that *B. clarridgeiae* might not be prevalent in Japan
In this study, it was found that PFGE was one of useful tools to differentiate of *B. henselae* strains and to investigate the epidemiology of *B. henselae* infection.

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