provides a better understanding of evolutional history of the organisms.

From the phylogenetic tree of the concatenated sequences, the strains were classified into two large groups according to geographical region: namely, an Asian group and an American/European group. All of the *B. grahamii* strains, except those from *Apodemus* mice, were also classified according to their host rodent genera in the phylogenetic analysis of both the concatenated sequences and the *gltA* sequences. However, *Apodemus* mice from Japan, China, and Russia were shown to harbor two different types of strain belonging to the Asian and the American/European group, indicating that *B. grahamii* are more accurately classified according to geographical region rather than to the host rodent genera. To confirm this hypothesis, it will be necessary to examine the genetic profiles of greater numbers of *B. grahamii* strains from many areas of the world.

In the phylogenetic analysis of the concatenated sequences, the Asian group consisted of strains from Japan and China, and the Japanese strains were divided into two subgroups, termed Japan 1 and Japan 2. The strains Ehime 1-1, Nakanoshima 10-1, and Tokushima 4-1 of subgroup Japan 2 were derived from the southern part of Japan and were more closely related to Chinese strains than to the strains of subgroup Japan 1. On the other hand, the phylogenetic analysis of *gltA* showed that the sequences of JP1-gltA were closely related to three sequences obtained from two *A. agrarius* hosts and an *A. peninsulae* host from Far Eastern Russia. On the basis of these phylogenetic analyses, we hypothesize that the *B. grahamii* strains distributed in wild rodents in Japan originated from two different geographic regions, i.e., China and Far Eastern Russia (Fig. 3). Furthermore, a sequence (AY584857) of *B. grahamii* from Far Eastern Russia was closely related to the sequences of *B. grahamii* from the USA. These findings suggest that Far Eastern Russia is one of the areas where *B. grahamii* strains of the Asian and American/European group might coexist (Fig. 3).

Phylogenetic analysis of the concatenated sequences showed that strains from the North American continent (Canada and the USA) and the European continent (the UK and Russia) were related to each other and formed a large clade, namely, the American/European group. In the phylogenetic analysis of *gltA*, a sequence (AF086637) of *B. grahamii* from *Mu. musculus* host in the USA was located outside the clades comprising strains from Canada, the UK, Russia, and the USA. Therefore, *B. grahamii* from *Mu. musculus* host in the USA is likely to be an ancestral lineage that previously branched away from the other strains of the American/European group. Furthermore, strict host specificity between *B. grahamii* and *My. gapperi* has been observed in Canada even though many rodent species exist there [17]. By contrast, *B. grahamii* strains in Europe have been isolated from a variety of host rodent species such as *A. uralensis* and *A. flavicollis* in Russia [28] and *A. sylvaticus*, *My. glareolus*, and *Mi. agrestis* in the UK [3]. These findings suggest the possibility that the geographic origin of *B. grahamii* strains of the American/European group is the North American continent rather than the European continent and that the organisms migrated with their rodent hosts and subsequently were distributed to many host rodent species in European countries. Therefore, we hypothesize that *B. grahamii* crossed the Bering Strait with their host rodents from the North American continent to Eurasia during the glacial epoch of the Pleistocene and may have been distributed through their ability to adapt to a wide range of host rodents (Fig. 3).

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References

1. Bereswill S, Hinkelmann S, Kist M, Sander A (1999) Molecular analysis of riboflavin synthesis genes in *Bartonella henselae* and use of the *ribC* gene for differentiation of *Bartonella* species by PCR. J Clin Microbiol 37:3159–3166
2. Bermond D, Heller R, Barrat F, Delacour G, Dehio C, Alliot A, Monteil H, Chomel B, Boulouis HJ, Piemont Y (2000) *Bartonella birtlesii* sp. nov., isolated from small mammals (*Apodemus* spp.). Int J Syst Evol Microbiol 50(Pt 6):1973–1979
3. Birtles RJ, Harrison TG, Molyneux DH (1994) *Grahamella* in small woodland mammals in the U.K. isolation, prevalence and host specificity. Ann Trop Med Parasitol 88:317–327
4. Birtles RJ, Harrison TG, Saunders NA, Molyneux DH (1995) Proposals to unify the genera *Grahamella* and *Bartonella*, with descriptions of *Bartonella talpae* comb. nov., *Bartonella peromysci* comb. nov., and three new species, *Bartonella grahamii* sp. nov., *Bartonella taylorii* sp. nov., and *Bartonella doshiae* sp. nov. Int J Syst Bacteriol 45:1–8
5. Brenner DJ, O'Connor SP, Winkler HH, Steigerwalt AG (1993) Proposals to unify the genera *Bartonella* and *Rochalimaea*, with descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb. nov., *Bartonella henselae* comb. nov., and *Bartonella elizabethae* comb. nov., and to remove the family *Bartonellaceae* from the order *Rickettsiales*. Int J Syst Bacteriol 43:777–786
6. Castle KT, Kosoy M, Lerdthusnee K, Phelan L, Bai Y, Gage KL, Leepitakrat W, Monkanna T, Khlaimanee N, Chandranoi K, Jones JW, Coleman RE (2004) Prevalence and diversity of *Bartonella* in rodents of northern Thailand: a comparison with *Bartonella* in rodents from southern China. Am J Trop Med Hyg 70:429–433
7. Chomel BB, Boulouis HJ, Maruyama S, Breitschwerdt EB (2006) *Bartonella* spp. in pets and effect on human health. Emerg Infect Dis 12:389–394
8. Daly JS, Worthington MG, Brenner DJ, Moss CW, Hollis DG, Weyant RS, Steigerwalt AG, Weaver RE, Daneshvar MI, O'Connor SP (1993) *Rochalimaea elizabethae* sp. nov. isolated from a patient with endocarditis. J Clin Microbiol 31:872–881

9. Ehrenborg C, Handley S, Ellis B, Mills J, Holmberg M (2003) *Bartonella grahamii* infecting rodents display high genetic diversity over short geographic distances. Ann N Y Acad Sci 990:233–235
10. Ellis BA, Regnery RL, Beati L, Bacellar F, Rood M, Glass GG, Marston E, Ksiazek TG, Jones D, Childs JE (1999) Rats of the genus *Rattus* are reservoir hosts for pathogenic *Bartonella* species: an Old World origin for a New World disease. J Infect Dis 180:220–224
11. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Évolution 39:783–791
12. Gundi VA, Davoust B, Khamis A, Boni M, Raoult D, La Scola B (2004) Isolation of *Bartonella rattimassiliensis* sp. nov. and *Bartonella phoceensis* sp. nov. from European *Rattus norvegicus*. J Clin Microbiol 42:3816–3818
13. Heller R, Artois M, Xemar V, De Briel D, Gehin H, Jaulhac B, Monteil H, Piemont Y (1997) Prevalence of *Bartonella henselae* and *Bartonella clarridgeiae* in stray cats. J Clin Microbiol 35:1327–1331
14. Heller R, Riegel P, Hansmann Y, Delacour G, Bermond D, Dehio C, Lamarque F, Monteil H, Chomel B, Piemont Y (1998) *Bartonella tribocorum* sp. nov., a new *Bartonella* species isolated from the blood of wild rats. Int J Syst Bacteriol 48(Pt 4):1333–1339
15. Holmberg M, Mills JN, McGill S, Benjamin G, Ellis BA (2003) *Bartonella* infection in sylvatic small mammals of central Sweden. Epidemiol Infect 130:149–157
16. Inoue K, Maruyama S, Kabeya H, Yamada N, Ohashi N, Sato Y, Yukawa M, Masuzawa T, Kawamori F, Kadosaka T, Takada N, Fujita H, Kawabata H (2008) Prevalence and genetic diversity of *Bartonella* species isolated from wild rodents in Japan. Appl Environ Microbiol 74:5086–5092
17. Jardine C, Appleyard G, Kosoy MY, McColl D, Chirino-Trejo M, Wobeser G, Leighton FA (2005) Rodent-associated *Bartonella* in Saskatchewan, Canada. Vector Borne Zoonotic Dis 5:402–409
18. Johnson G, Ayers M, McClure SC, Richardson SE, Tellier R (2003) Detection and identification of *Bartonella* species pathogenic for humans by PCR amplification targeting the riboflavin synthase gene (*ribC*). J Clin Microbiol 41:1069–1072
19. Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HN (ed) Mammalian protein metabolism. Academic, New York, pp 21–132
20. Kerkhoff FT, Bergmans AM, van Der Zee A, Rothova A (1999) Demonstration of *Bartonella grahamii* DNA in ocular fluids of a patient with neuroretinitis. J Clin Microbiol 37:4034–4038
21. Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120
22. Kordick DL, Swaminathan B, Greene CE, Wilson KH, Whitney AM, O'Connor S, Hollis DG, Matar GM, Steigerwalt AG, Malcolm GB, Hayes PS, Hadfield TL, Breitschwerdt EB, Brenner DJ (1996) *Bartonella vinsonii* subsp. *berkhoffii* subsp. nov., isolated from dogs; *Bartonella vinsonii* subsp. *vinsonii*; and emended description of *Bartonella vinsonii*. Int J Syst Bacteriol 46:704–709
23. Kosoy M, Murray M, Gilmore RD Jr, Bai Y, Gage KL (2003) *Bartonella* strains from ground squirrels are identical to *Bartonella washoensis* isolated from a human patient. J Clin Microbiol 41:645–650
24. Kosoy MY, Regnery RL, Tzianabos T, Marston EL, Jones DC, Green D, Maupin GO, Olson JG, Childs JE (1997) Distribution, diversity, and host specificity of *Bartonella* in rodents from the Southeastern United States. Am J Trop Med Hyg 57:578–588
25. Kosoy MY, Saito EK, Green D, Marston EL, Jones DC, Childs JE (2000) Experimental evidence of host specificity of *Bartonella* infection in rodents. Comp Immunol Microbiol Infect Dis 23:221–238
26. Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform 5:150–163
27. La Scola B, Zeaiter Z, Khamis A, Raoult D (2003) Gene-sequence-based criteria for species definition in bacteriology: the *Bartonella* paradigm. Trends Microbiol 11:318–321
28. Markov AP, Lopyrev IV, Irkhin AI, Khliap LA, Levitskii SA, Kirillov MIu, Manuvera VA, Il'ina TS, Pokrovskaia MS, Aleshkin GI, Bashkirov VN, Smirnov GB, Kosoi M (2006) [Wild small mammals are the reservoir hosts of the *Bartonella* genus bacteria in the south of Moscow region]. Mol Gen Mikrobiol Viruso l:8–13
29. Mediannikov O, Ivanov L, Zdanovskaya N, Vysochina N, Fournier PE, Tarasevich I, Raoult D (2005) Molecular screening of *Bartonella* species in rodents from the Russian Far East. Ann N Y Acad Sci 1063:308–311
30. Norman AF, Regnery R, Jameson P, Greene C, Krause DC (1995) Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. J Clin Microbiol 33:1797–1803
31. Renesto P, Gouvernet J, Drancourt M, Roux V, Raoult D (2001) Use of *rpoB* gene analysis for detection and identification of *Bartonella* species. J Clin Microbiol 39:430–437
32. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
33. Tea A, Alexiou-Daniel S, Papoutsi A, Papa A, Antoniadis A (2004) *Bartonella* species isolated from rodents, Greece. Emerg Infect Dis 10:963–964
34. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882
35. Welch DF, Carroll KC, Hofmeister EK, Persing DH, Robison DA, Steigerwalt AG, Brenner DJ (1999) Isolation of a new subspecies, *Bartonella vinsonii* subsp. *arupensis*, from a cattle rancher: identity with isolates found in conjunction with *Borrelia burgdorferi* and *Babesia microti* among naturally infected mice. J Clin Microbiol 37:2598–2601
36. Ying B, Kosoy MY, Maupin GO, Tsuchiya KR, Gage KL (2002) Genetic and ecologic characteristics of *Bartonella* communities in rodents in southern China. Am J Trop Med Hyg 66:622–627
37. Zeaiter Z, Fournier PE, Ogata H, Raoult D (2002) Phylogenetic classification of *Bartonella* species by comparing *groEL* sequences. Int J Syst Evol Microbiol 52:165–171
38. Zeaiter Z, Liang Z, Raoult D (2002) Genetic classification and differentiation of *Bartonella* species based on comparison of partial *ftsZ* gene sequences. J Clin Microbiol 40:3641–3647

Molecular Epidemiology of Feline and Human *Bartonella henselae* Isolates

Rim Bouchouicha, Benoit Durand, Martine Monteil, B.B. Chomel, Moez Berrich, Mardjan Arvand, Richard J. Birtles, Edward B. Breitschwerdt, Jane E. Koehler, Ricardo Maggi, Soichi Maruyama, Rick Kasten, Elisabeth Petit, Henri-Jean Boulouis, and Nadia Haddad

Multiple locus variable number tandem repeat analysis was performed on 178 *Bartonella henselae* isolates from 9 countries; 99 profiles were distributed into 2 groups. Human isolates/strains were placed into the second group. Genotype I and II isolates shared no common profile. All genotype I isolates clustered within group B. The evolutive implications are discussed.

Bartonella henselae is the zoonotic agent of cat-scratch disease and has been associated with bacillary angiomatosis, bacillary peliosis, endocarditis, osteomyelitis, and neuroretinitis (*1*). It is usually present in low numbers in infected human tissues, whereas cats, the natural reservoir for the bacterium, are prone to persistent bacteremia. Therefore, most *B. henselae* isolates are of feline origin. Two genotypes, based on 16S rDNA differences, have been described (*1*). Genotype I was more frequently observed in humans in some countries where most cats are infected with genotype II (*2,3*). Genotype II is more prevalent in cats in Europe, Australia, and the United States (*2–6*), and most feline isolates from Asia belong to genotype I (*7–9*). In a recent study, 3 (1.6%) of 191 *B. henselae* isolates harbored 2 different 16S rDNA copies and could not be assigned to a distinct genotype (*10*). However, most isolates harbored 2 identical 16S rDNA copies and were assigned to either type I or II, confirming that delineation of *B. henselae* isolates in two 16S rDNA types is generally reasonable.

Author affiliations: Ecole Nationale Vétérinaire d'Alfort, Maisons Alfort, France (R. Bouchouicha, M. Monteil, M. Berrich, E. Petit, H.-J. Boulouis, N. Haddad); Agence Française de Sécurité Sanitaire des Aliments, Maisons Alfort (B. Durand); University of California, Davis, California, USA (B. Chomel, R. Kasten); Zentrum für Gesundheitsschutz, Dillenburg, Germany (M. Arvand); University of Liverpool, Cheshire, UK (R.J. Birtles); North Carolina State University, Raleigh, North Carolina, USA (E. Breitschwerdt, R. Maggi); University of California, San Francisco, California, USA (J. Koehler); and Nihon University, Kanagawa, Japan (S. Maruyama)

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Multiple-locus variable number tandem repeat analysis (MLVA) was recently developed for *B. henselae* typing (*6*). The results are produced in an intrinsically quantitative form, called a profile, corresponding to the number of basic units in an isolate for each variable number tandem repeat (VNTR). MLVA was more discriminatory (*11*) than the other widely used typing techniques, such as pulsed-field gel electrophoresis (*2*), multilocus sequence typing (MLST) (*12*) and multispacer typing (MST) (*13*). In our protocol, MLVA involves the amplification of 5 main VNTR loci, BHV-A to -E, for *B. henselae* VNTRs (*6*).

The Study

We analyzed 178 *B. henselae* isolates/strains from various sources (Table 1): 156 (88%) feline isolates/strains, 21 (11%) from diseased humans, and 1 isolate from a sick dog. The number of alleles varied from 7 (BHV-E) to 22 (BHV-B). Most of the European isolates (all but 1 of feline origin) (*2,4,6*) and of the American isolates/strains (North Carolina and California) (*5,14*), of which 85% were of feline origin, belonged to genotype II (89% and 64.6%, respectively). The Asian isolates (all but 1 of feline origin) (*7–9*) and the Australasian isolates (60% of human origin) (*12*), mainly belonged to genotype I (89.6% and 65%, respectively).

Ninety-nine different MLVA profiles were observed (Table 1), corresponding to an average number of isolates per profile of 1.8 (Table 2). Sixty-nine of these profiles were found in only 1 isolate or strain (67%), and 30 were observed in >1 isolate. Among these, none was shared by genotype I and genotype II isolates. Diversity index (DI) was 0.98 (Table 1). Diversity was observed in both genotypes because genotype-specific DIs were almost identical (Table 1).

MLVA profiles appeared location-specific because only 4 (13%) of the 30 profiles observed in >1 isolate/strain were present in >1 continent (Table 2). Within continents, no marked dominance of a given profile was observed, and continent-specific DIs were similar (Table 1).

Of the 99 *B. henselae* profiles, 12 were obtained from the 21 human isolates/strains and 1 from the dog, whereas 92 profiles were obtained from the 156 feline isolates. Five profiles were common to 5 human and 11 feline isolates. Among the 30 profiles observed in ≥2 isolates, 23 were observed only in feline isolates (Table 2). The proportion of genotype I profiles was significantly higher in human-specific profiles than in cat-specific profiles ($p = 0.01$, by Fisher test).

Table 1. Description of *Bartonella henselae* isolates and strains tested, global diversity of the typing system, and diversity variations according to 16S rDNA genotype, continent, and host*

Characteristics	No. isolates/ strains	No. profiles	Average no. isolates/profiles	DI	No. alleles (minimum–maximum no. repeats)				
					A	B	C	D	E
All *B. henselae*	178	99	1.8	0.98	8 (9–16)	22 (5–37)	14 (1–25)	8 (1–9)	7 (1–7)
16S rDNA genotype I	64	44	1.5	0.98	6	12	11	6	6
16S rDNA genotype II	114	55	2.0	0.97	6	15	7	7	6
Location									
Europe	80†	42	1.9	0.95	6	12	6	6	5
Asia	29‡	22	1.3	0.98	7	10	8	4	3
USA	49§	28	1.7	0.95	4	12	7	6	6
Australia–New Zealand	20¶	11	1.8	0.87	4	5	5	4	6
Host									
Human + dog	22#	12	1.8	0.87	2	6	6	3	6
Healthy cat	156	92	1.7	0.98	8	20	11	8	7

*DI, diversity index; A, BHV-A; B, BHV-B; C, BHV-C; D, BHV-D; E, BHV-E.
†Denmark, 18 (*7*); France, 23 (*7*); Germany, 27 (*2*); UK, 12 (*5*).
‡Japan, 12 (*8*); Philippines, 7 (*9*); Thailand, 10 (*10*).
§California: 36, including 5 owners and their 11 cats (*6,14*); North Carolina, 12 (provided by Ed Breitschwerdt). Reference strain Houston 1.
¶Australia, 18 and New Zealand, 2 (*3,12*).
#21 human isolates from 1 German patient with bacillary angiomatosis (BA) (*2*), 1 Japanese patient with cat-scratch disease (provided by S. Maruyama), 12 Australian human patients with cat-scratch disease (*3*), 5 California human patients with BA (*14*), 1 North Carolina patient with a wide range of symptoms, including fatigue, joint pain, insomnia, headache, blurred vision, irritability (provided by Ed Breitschwerdt), plus the reference strain Houston 1 (ATCC 49882, initially isolated from a patient with BA) (*3*), and 1 isolate from a dog with endocarditis (provided by Ed Breitschwerdt).

For BHV-A, only 2 alleles (14 and 15 copies) were found in isolates from humans, whereas all 8 identified alleles were observed in cat isolates. The number of repeats differed significantly between sick humans and healthy cats ($p = 0.02$, by Fisher test).

Relationships between the 99 MLVA profiles were analyzed by unweighted pair group method with arithmatic mean (UPGMA), using a categorical distance, with a *B. koehlerae* isolate used as an outgroup. To take into account that UPGMA is sensitive to taxa entry order, we computed the majority-rule consensus tree of 500 dendrograms built with random taxa entry order. MLVA profiles were grouped into 2 main groups named A and B (online Appendix Figure, available from www.cdc.gov/EID/content/15/5/813.htm). Group A (26 profiles), was exclusively constituted by genotype II feline isolates. Group B (73 profiles), to which all human isolates belonged, further divided in 2 subgroups, Ba and Bb. Subgroup Ba (38 profiles) was exclusively composed of genotype I isolates, including the reference strain Houston 1 and a homogenous subgroup, Ba1, containing 84% of the Asian isolates. Finally, 83% of subgroup Bb isolates belonged to genotype II (29/35 profiles).

The utility of MLVA for molecular epidemiologic analysis of clusters was tested using isolates from California cats and their owners (*14*). Five human–cat groups of *B. henselae* isolates were analyzed. For 1 cat-human pair of isolates, which belonged, respectively, to genotype II and genotype I, major profile differences were observed, as expected. The 4 other cat-human groups, which possessed the same genotype, also had the same MLVA profile with the 5 tested BHV, as well as with the 6 additional BHV (F–K) and variant alleles for BHV-A and/or B (*6*). Sequencing confirmed these results.

Conclusions

Our results confirm that VNTRs are excellent molecular markers for confirming or excluding the responsibility of a given cat in the transmission of *B. henselae* to a human. In California, the profile identity observed within 4 clusters further supports the hypothesis that all these humans acquired infection from their respective domestic cat contacts.

MLVA enabled a clear separation between genotypes I and II, because no profile was shared between both genotypes. The dendrogram showed a high level of discrimination between 16S rDNA genotypes in the *B. henselae* population tested. Interestingly, the groups and subgroups delineated by MLVA were the same as those defined by MLST, a standard method for phylogenetic analysis (*12*). The same was observed with MST (*13*). The isolates of the subgroup Bb appeared divergent and distant from each other and from subgroup Ba that contains almost all genotype I profiles (98%). Moreover and despite possible clustering for some of the isolates, none of the 21 human isolates was present in group A. Interestingly, as for most of the human patients, the isolate obtained from the ill dog also belonged to genotype I.

These observations suggest that all genotype I isolates could be phylogenetically derived from genotype II isolates located in group B but not in group A, as already suggested using MLST (*15*). This observation could mean that genotype II isolates belonging to group B are closer to genotype I isolates than to genotype II isolates belonging to group

Table 2. Distribution of *Bartonella henselae* isolates/strains by 16S rDNA genotype, host, and location for profiles with ≥2 isolates*

VNTR profile					No.	16S rDNA genotype		Host		Location			
A	B	C	D	E	isolates	I	II	Human	Healthy cat	Europe	Asia	USA	Aus-NZ
10	14	2	2	1	14		14		14	14			
9	15	2	1	1	8		8		8	5		3	
10	15	2	2	1	8		8		8	8			
14	34	2	7	4	8		8	1	7			8	
14	22	10	5	3	7	7		7					7
14	32	8	7	4	6		6	2	4			6	
14	20	10	7	5	4	4		2	2	3		1	
9	14	2	2	1	4		4		4	4			
10	15	2	1	1	4		4		4	4			
15	20	10	8	2	4	4			4		4		
13	14	6	5	4	3		3		3	3			
15	20	10	8	4	3	3		1	2			3	
13	31	6	5	5	2	2			2	2			
9	14	2	1	1	2		2		2	1		1	
9	15	2	2	1	2		2		2	2			
13	34	10	8	3	2	2			2		2		
14	36	8	7	4	2		2		2			2	
13	32	8	7	4	2		2		2			2	
9	15	2	1	3	2		2		2		1	1	
14	32	8	7	1	2		2	2					2
14	26	6	8	4	2		2		2				2
15	32	10	8	5	2	2			2				2
14	11	6	7	4	2		2		2	2			
13	20	7	8	2	2	2			2			2	
14	20	6	1	2	2	2		1	1			2	
10	15	3	1	1	2		2		2	2			
10	15	3	2	1	2		2		2	2			
14	20	10	8	2	2	2			2		2		
14	18	10	1	3	2	2			2		2		
15	20	10	1	2	2	2			2		2		

*VNTR, variable number tandem repeat; Aus-NZ, Australia and New Zealand; A, BHV-A; B, BHV-B; C, BHV-C; D, BHV-D; E, BHV-E.

A; it also raises an important clinical question: Are feline genotype II isolates belonging to group A nonpathogenic for humans? Genotype I isolates could represent the most pathogenic isolates for humans within a group of potentially zoonotic isolates, all belonging to group B and could represent an ultimate evolutionary step toward human infection. Additionally, within group B, the differences in the number of BHV-A repeat units observed between isolates from patients (humans, dog) versus cat isolates suggest that this specific VNTR could constitute a marker for the ability to cross the species barrier from reservoir cats to susceptible species, independent of the 16S rDNA genotype.

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Ms Bouchouicha is a third-year doctoral student at the Unité Mixte de Recherche, Biologie Moléculaire et Immunologie Parasitaires et Fongiques, Maison-Alfort, France. Her research interests focus on the molecular epidemiology of animal diseases and zoonoses.

References

1. Boulouis HJ, Chang CC, Henn JB, Kasten RW, Chomel BB. Factors associated with the rapid emergence of zoonotic *Bartonella* infections. Vet Res. 2005;36:383–410. DOI: 10.1051/vetres:2005009
2. Arvand M, Klose AJ, Schwartz-Porsche D, Hahn H, Wendt C. Genetic variability and prevalence of *Bartonella henselae* in cats in Berlin, Germany, and analysis of its genetic relatedness to a strain from Berlin that is pathogenic for humans. J Clin Microbiol. 2001;39:743–6. DOI: 10.1128/JCM.39.2.743-746.2001
3. Dillon B, Valenzuela J, Don R, Blanckenberg D, Wigney DI, Malik R, et al. Limited diversity among human isolates of *Bartonella henselae*. J Clin Microbiol. 2002;40:4691–9. DOI: 10.1128/JCM.40.12.4691-4699.2002
4. Birtles RJ, Laycock G, Kenny MJ, Shaw SE, Day MJ. Prevalence of *Bartonella* species causing bacteraemia in domesticated and companion animals in the United Kingdom. Vet Rec. 2002;151:225–9.

5. Chomel BB, Abbott RC, Kasten RW, Floyd-Hawkins KA, Kass PH, Glaser CA, et al. *Bartonella henselae* prevalence in domestic cats in California: risk factors and association between bacteremia and antibody titers. J Clin Microbiol. 1995;33:2445–50.
6. Monteil M, Durand B, Bouchouicha R, Petit E, Chomel B, Arvand M, et al. Development of discriminatory multiple-locus variable-number tandem repeat analysis for *Bartonella henselae* Microbiol. 2007;153:1141–8.
7. Maruyama S, Nakamura Y, Kabeya H, Tanaka S, Sakai T, Katsube Y. Prevalence of *Bartonella henselae, Bartonella clarridgeiae* and the 16S rRNA gene types of *Bartonella henselae* among pet cats in Japan. J Vet Med Sci. 2000;62:273–9. DOI: 10.1292/jvms.62.273
8. Chomel BB, Carlos ET, Kasten RW, Yamamoto K, Chang CC, Carlos RS, et al. *Bartonella henselae* and *Bartonella clarridgeiae* infection in domestic cats from the Philippines. Am J Trop Med Hyg. 1999;60:593–7.
9. Maruyama S, Sakai T, Morita Y, Tanaka S, Kabeya H, Boonmar S, et al. Prevalence of *Bartonella* species and 16S rRNA gene types of *Bartonella henselae* from domestic cats in Thailand. Am J Trop Med Hyg. 2001;65:783–7.
10. Viezens J, Arvand, M. Simultaneous presence of two different copies of the 16S rRNA gene in *Bartonella henselae*. Microbiology. 2008;154:2881–6. DOI: 10.1099/mic.0.2008/018630-0
11. Bouchouicha R, Boulouis H-J, Berrich M, Monteil M, Chomel B, Haddad N. Comparison of the performances of MLVA versus the main other typing techniques for *Bartonella henselae*. Clin Microbiol Infect. In press.
12. Iredell J, Blanckenberg D, Arvand M, Grauling S, Feil EJ, Birtles RJ. Characterization of the natural population of *Bartonella henselae* by multilocus sequence typing. J Clin Microbiol. 2003;41:5071–9. DOI: 10.1128/JCM.41.11.5071-5079.2003
13. Li W, Raoult D, Fournier PE. Genetic diversity of *Bartonella henselae* in human infection detected with multispacer typing. Emerg Infect Dis. 2007;13:1178–83.
14. Chang CC, Chomel BB, Kasten RW, Tappero JW, Sanchez MA, Koehler JE. Molecular epidemiology of *Bartonella henselae* infection in human immunodeficiency virus-infected patients and their cat contacts, using pulsed-field gel electrophoresis and genotyping. J Infect Dis. 2002;186:1733–9. DOI: 10.1086/345764
15. Arvand M, Feil EJ, Giladi M, Boulouis HJ, Viezens J. Multi-locus sequence typing of *Bartonella henselae* isolates from three continents reveals hypervirulent and feline-associated clones. PLoS One. 2007;2:e1346. DOI: 10.1371/journal.pone.0001346

Address for correspondence: Nadia Haddad, Ecole Nationale Vétérinaire d'Alfort, 7, Av du Général de Gaulle, 94704 Maisons Alfort CEDEX, France; email: nhaddad@vet-alfort.fr

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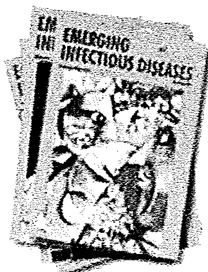

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ORIGINAL ARTICLE

A novel multiplex PCR assay for *Salmonella* subspecies identification

K. Lee, T. Iwata, M. Shimizu, T. Taniguchi, A. Nakadai, Y. Hirota and H. Hayashidani

Division of Animal Life Science, Institute of Symbiotic Science and Technology, Tokyo University of Agriculture and Technology, Tokyo, Japan

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Correspondence
Hideki Hayashidani, Division of Animal Life Science, Institute of Symbiotic Science and Technology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan.
E-mail: eisei@cc.tuat.ac.jp

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Abstract

Aim: To develop a novel multiplex polymerase chain reaction (PCR) assay with six primer pairs for *Salmonella* subspecies identification.

Methods and Results: Five primer pairs were chosen to detect the genes (*fljB*, *mdcA*, *gatD*, *stn* and STM4057) responsible for several phenotypic traits or encoding (sub) species-specific regions. A primer pair for *invA* was added to simultaneously detect *Salmonella*. The combination of these primer pairs was expected to give unique results to all subspecies, including *Salmonella bongori*. The multiplex PCR assay was optimized and evaluated with 53 *Salmonella* strains representing all *S. enterica* subspecies, *S. bongori* and five non-*Salmonella* strains. The multiplex PCR assay revealed that the genotypes were well correlated with the phenotypes in the *Salmonella* strains tested. The unique band patterns to their subspecies were generated from 94·3% (50/53) of the *Salmonella* strains, and no product from other strains by the multiplex PCR assay.

Conclusions: The multiplex PCR assay we developed was found to be a rapid, specific and easy to perform method compared with traditional biochemical tests for *Salmonella* subspecies identification, especially for rapid screening of large numbers of samples.

Significance and Impact of the Study: The assay will be useful for characterizing *Salmonella* isolates from reptiles, which belong to various subspecies, and therefore add to the scientific understanding of reptile-associated Salmonellosis.

Introduction

Salmonella is one of the most important human and animal gastrointestinal pathogens in the world (Bäumler *et al.* 2000; Bopp *et al.* 2003; D'Aoust and Maurer 2007). The genus *Salmonella* belongs to the family Enterobacteriaceae and is divided taxonomically into two species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* comprises six subspecies: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* (VI) (Tindall *et al.* 2005). However, for simplicity, *S. bongori* is still commonly referred to as subsp. V. Recently, Shelobolina *et al.* (2004) proposed *Salmonella subterranean*. But strains of this species have been isolated only from a low-pH, nitrate- and U (VI)-contaminated subsurface sediment and not from human or other animals. *Salmonella* is classified into more than 2500 serovars using the Kauffmann–White scheme (Popoff 2001). Most of the isolates from human gastroenteritis patients and warm-blooded vertebrates belong to *S. enterica* subsp. I., while other subspecies strains and *S. bongori* are usually isolated from cold-blooded vertebrates and the environment (Bopp *et al.* 2003).

Recently, the increase in exotic pet reptile popularity has led to an increase in the number of reptile-associated Salmonellosis (Centers for Disease Control and Prevention, CDC 2003). Clinical cases of reptile-associated Salmonellosis have been reported worldwide (Sanyal *et al.* 1997; Woodward *et al.* 1997; Römkens *et al.* 2003; De Jong *et al.* 2005; Kaibu *et al.* 2006). *Salmonella*

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originating from reptiles belong to various subspecies. Although *S. enterica* subsp. I is also common among them, more than 30% of the isolates belong to other subspecies (Geue and Loschner 2002; Nakadai *et al.* 2005; Pasmans *et al.* 2005). Moreover, previous studies have indicated that such uncommon *Salmonella* subspecies are sporadically but continuously isolated from the environment and foods (Heinzerling and Bockemuhl 1996; Giammanco *et al.* 2002; Tavechio *et al.* 2002). Subspecies identification is helpful for characterizing such uncommon *Salmonella* isolates from reptiles or the environment, because serotyping is laborious and it is not realistic to always have such uncommon factor antiserum (e.g. O:50–67) ready in many laboratories (Popoff and Le Minor 2005). Classification based on biochemical characteristics is the accepted method to identify *Salmonella* subspecies (Popoff and Le Minor 2005). However, performing each biochemical test for the classification is labour intensive and time-consuming. In addition, commercial identification kits cannot always distinguish all *Salmonella* subspecies (O'Hara 2005). However, there are few studies published that describe an alternative or complementary method for identifying *Salmonella* at subspecies level.

In *S. enterica* subsp. I, several studies have been published that describe identifications at serovar level using multiplex polymerase chain reaction (PCR) assays (Herrera-León *et al.* 2004; Kim S. *et al.* 2006). In addition to the higher throughput than conventional PCR assays, a multiplex PCR assay requires access to only a thermal cycler and minigel electrophoresis apparatus. Therefore, the assay still remains an important accessible method for many laboratories. In *S. enterica* subspecies other than subsp. I, a lot of sequence data have been published recently. It would be useful to develop a practical multiplex PCR assay for *Salmonella* identification at subspecies level using those data.

In the present study, we report on the development of a simple multiplex PCR assay for the rapid, reliable and easy identification of all *Salmonella* subspecies, including *S. bongori*. This method is based on the amplification of the genes responsible for several phenotypic traits or encoding (sub) species-specific genes.

Materials and methods

Bacterial strains

All 53 strains used in the present study are shown in Table 1. These strains represent all *Salmonella* subspecies, including *S. bongori*. *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus aureus* were used to verify the specificity of the multiplex PCR assay and were obtained from the Japan Collection of Microorganisms (JCM, Saitama, Japan). Overnight culture on trypticase soy agar (Becton Dickinson, Franklin Lakes, NJ, USA) of all strains were suspended in skim milk (Becton Dickinson) and stored at −80°C until analysis (Anon 2004).

Extraction of template DNA for PCR assay

All strains were plated on trypticase soy agar (Becton Dickinson) with overnight incubation at 37°C. Bacterial genomic DNA was then extracted after growth overnight in Luria-Bertani broth (Difco Laboratories, Detroit, MI, USA) by using the Wizard® genomic DNA purification kit (Promega Corp., Madison, WI, USA) and stored at 4°C.

Primer design

Target genes were chosen for their ability to give unique results to identify all *Salmonella* subspecies, including *S. bongori* (Table 2). Primer pairs mdcA 7–8, gatD P5–P6 and stn f1–r1 were designed with Primer3 software (Whitehead Institute, Cambridge, MA, USA). All primer pairs were synthesized and supplied by SIGMA Genosys Japan (Hokkaido, Japan).

The *fljB* gene encodes phase 2 flagellin and a primer pair fljB 1–2 was used for differentiation between monophasic and diphasic subspecies, except *S. bongori*. Although *S. bongori* is a monophasic species, the *fljB* gene has been detected in a previous study (Bäumler and Heffron 1998). The *mdcA* and *gatD* genes were chosen because these genes contribute to phenotypic traits that are useful for differentiating among *Salmonella* subspecies. The *mdcA* gene encodes the alpha subunit of the malonate decarboxylase (MdcA) and MdcA contributes to malonate utilization. The *gatD* gene encodes the galactitol-1-phosphate dehydrogenase (GatD) and GatD contributes to acid production from galactitol (synonym of dulcitol) (Miyamoto *et al.* 1999). More than 90% of *S. enterica* subsp. II, IIIa and IIIb utilize malonate and more than 90% of *S. enterica* subsp. I, II and *S. bongori* produce acid from galactitol (Popoff and Le Minor 2005). As the presence and sequence of the *mdcA* gene has not been reported in *Salmonella*, the *mdcA* sequence of *K. pneumoniae* (accession no. U95087) was compared with the sequence data of *S. enterica* subsp. IIIa and IIIb from Washington University (http://genome.wustl.edu/home.cgi) using Basic Local Alignment Search Tool (BLAST). Both strains had high homology (84% in DNA sequence and 92% in protein sequence) with *mdcA* of *K. pneumoniae*. Then, we constructed a primer pair mdcA 7–8 based on the sequence of the putative *mdcA* of *S. enterica* subsp. IIIa and IIIb. A primer pair gatD P5–6

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Table 1 Bacterial strains in the present study and results by monoplex and multiplex polymerase chain reaction assay

Species	Subspecies	Strain	Serovar	Source	Monoplex PCR result						Multiplex PCR pattern
					fljB	*mdcA*	*gatD*	*stn*	STM4057	*invA*	
Salmonella enterica	*enterica* (I)	NMJS1	Typhimurium	Black rat	+	−	+	+	+	+	I
		JCM1652	Enteritidis	Unknown	−	−	+	+	+	+	I
		R16D1	Minnesota	Turtle	+	−	+	+	+	+	I
		R19D1	Panama	Snake	+	−	+	+	+	+	I
		R22D1	Amsterdam	Lizard	+	−	+	+	+	+	I
		R10M1	Newport	Snake	+	−	+	+	+	+	I
		HDD6-1	Kentucky	Lizard	+	−	+	+	+	+	I
		R30M2	Muenchen	Lizard	+	−	+	+	+	+	I
		R45M1	Beaudesert	Lizard	+	−	+	+	+	+	I
		R10D1	Montevideo	Turtle	−	−	+	+	+	+	I
	salamae (II)	R42D2	13, 22:z_{29}: 1, 5	Turtle	+	+	+	+	−	+	II
		Kn127M1	1, 4,12, 27:b:-	Snake	−	+	+	+	+	+	UT
		R11D4	UT	Snake	+	+	+	+	−	+	II
		R21D2	UT	Lizard	+	−	+	−	−	+	V
		R42D3	UT	Turtle	+	+	+	+	−	+	II
		R52D1	UT	Lizard	+	−	+	+	−	+	VI
		R55D1	UT	Lizard	+	+	+	+	−	+	II
		R78D1	UT	Lizard	+	+	+	+	−	+	II
		R81D1	UT	Lizard	+	+	+	+	−	+	II
		R95D1	UT	Lizard	+	+	+	+	−	+	II
	arizonae (IIIa)	8M1	UT	Snake	−	+	−	+	−	+	IIIa
		Kn114D1	UT	Snake	−	+	−	+	−	+	IIIa
		HD5-2	UT	Snake	−	+	−	+	−	+	IIIa
		HDD5-3	UT	Snake	−	+	−	+	−	+	IIIa
		R56D1	UT	Snake	−	+	−	+	−	+	IIIa
		27M2	11:z4, z23:-	Lizard	−	+	−	+	−	+	IIIa
		156D1	UT	Snake	−	+	−	+	−	+	IIIa
		394D1	UT	Snake	−	+	−	+	−	+	IIIa
		451D1	UT	Turtle	−	+	−	+	−	+	IIIa
		453B1	UT	Snake	−	+	−	+	−	+	IIIa
	diarizonae (IIIb)	WC1D1	UT	Snake	+	+	−	+	−	+	IIIb
		WC6D1	UT	Snake	+	+	−	+	−	+	IIIb
		WC8M1	UT	Snake	+	+	−	+	−	+	IIIb
		WC19M1	UT	Snake	+	+	−	+	−	+	IIIb
		WC20D1	UT	Snake	+	+	−	+	−	+	IIIb
		R14M1	UT	Snake	+	+	−	+	−	+	IIIb
		R18M1	UT	Snake	+	+	−	+	−	+	IIIb
		R28D3	UT	Turtle	+	+	−	+	−	+	IIIb
		R48D1	UT	Snake	+	+	−	+	−	+	IIIb
		R54D1	UT	Snake	+	+	−	+	−	+	IIIb
	houtenae (IV)	7D1	UT	Snake	−	−	−	+	−	+	IV
		10D1	UT	Lizard	−	−	−	+	−	+	IV
		15D1	11:z4, z23:-	Lizard	−	−	−	+	−	+	IV
		26M1	11:z4, z23:-	Lizard	−	−	−	+	−	+	IV
		303E	UT	Lizard	−	−	−	+	−	+	IV
		R56M1	UT	Snake	−	−	−	+	−	+	IV
		R58D1	UT	Lizard	−	−	−	+	−	+	IV
		R75D1	UT	Lizard	−	−	−	+	−	+	IV
		263D1	UT	Snake	−	−	−	+	−	+	IV
		462D1	UT	Lizard	−	−	−	+	−	+	IV
	indica (VI)	CCUG30038	1, 6, 14, 25:a:e, n, x	Unknown	+	−	+	+	−	+	VI
Salmonella bongori (V)		321E	UT	Lizard	+	−	+	−	−	+	V
		321M	UT	Lizard	+	−	+	−	−	+	V

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Table 1 (*Continued*)

Species	Subspecies	Strain	Serovar	Source	Monoplex PCR result						Multiplex PCR pattern
					fljB	*mdcA*	*gatD*	*stn*	STM4057	*invA*	
Escherichia coli		JCM5491			–	–	–	–	–	–	–
Klebsiella pneumoniae		JCM1662			–	–	–	–	–	–	–
Pseudomonas aeruginosa		JCM6119			–	–	–	–	–	–	–
Enterococcus faecalis		JCM7783			–	–	–	–	–	–	–
Staphylococcus aureus		JCM20624			–	–	–	–	–	–	–

+, PCR product of expected size; –, no PCR product.
UT, untypable.

Table 2 Oligonucleotide primers and expected band patterns of each *Salmonella* species or subspecies

Primer	Sequence (5′–3′)	Target gene	Gene function	Product size(bp)	Reference	*Salmonella* species or subspecies						
						I	II	IIIa	IIIb	IV	VI	V
fljB 1	GACTCCATCCAGGCTGAAATCAC	*fljB*	Phase 2 flagellin	848	Baumler and Heffron	d	d	–	+	–	+	+
fljB 2	CGGCTTTGCTGGCATTGTAG											
mdcA 7	GGATGTACTCTTCCATCCCCAGT	*mdcA*	Putative malonate decarboxylase	728	This study	–	+	+	+	–	–	–
mdcA 8	CGTAGCGAGCATCTGGATATCTTT											
gatD P5	GGCGCCCATTATTATCCTATTAC	*gatD*	Galactitol-1-phosphate dehydrogenase	501	This study	+	+	–	–	–	d	+
gatD P6	CATTTCCCGGCTATTACAGGTAT											
stn f1	CGATCCCTTTCCCGCTATC	*stn*	*Salmonella* enterotoxin	179	This study	+	+	+	+	+	+	–
stn r1	GGCGAATGAGACGCTTAAG											
STM4057 f1	GGTGGCCTCGATGATTCCCG	STM4057	Putative inner membrane protein	137	Kim H. *et al.* (2006)	+	–	–	–	–	–	–
STM4057 r1	CCCACTTGTAGCGAGCGCCG											
INVA-1	ACAGTGCTCGTTTACGACCTGAAT	*invA*	Invasion protein	244	Chiu and Ou	+	+	+	+	+	+	+
INVA-2	AGACGACTGGTACTGATCGATAAT											

+, PCR product of expected size; –, no PCR product; d, differs among strains.

was constructed according to the previously reported *gatD* sequence (Miyamoto *et al.* 1999). Primer pairs for the *stn* and STM4057 genes were chosen for species and subspecies differentiation among *Salmonella*, respectively. The *stn* gene encodes *Salmonella* enterotoxin and is specific for *S. enterica* (Prager *et al.* 1995). However, alignment of *stn* sequences has shown that *S. bongori* had 88% sequence identity with *S. enterica* (Moore and Feist 2007). To distinguish these species, we have constructed the primer based on the variable region, in which five bases are replaced, for 3′ end of the primer stn r1 (position 971–975 in the *stn* gene; GenBank accession no. L16014). The STM4057 gene encodes putative inner membrane protein and a previous study has suggested it to be the *S. enterica* subsp. I specific gene (Kim H. *et al.* 2006). In addition to primer pairs for subspecies identification, a primer pair INVA 1–2 was used for simultaneous identification of *Salmonella* at the genus level (Chiu and Ou 1996). The *invA* gene has been commonly used to detect *Salmonella* (Malorny *et al.* 2003). These six primer pairs were expected to give unique results to each subspecies as shown in Table 2.

Multiplex PCR condition

Initially, we performed a monoplex PCR assay using each primer pair and observed the distribution of target genes among *Salmonella* subspecies. After validation of each primer pair, we combined them and confirmed that each amplification product was the correct size. Then we optimized the condition of the multiplex PCR assay.

Each multiplex PCR tube contained 0·3 mmol l^{-1} each deoxyribonucleotide triphosphate, 1·1 × Ex Taq Buffer (Takara Bio Inc., Shiga, Japan), 0·75 μmol l^{-1} each stn primer pair, 0·50 μmol l^{-1} each fljB, mdcA, gatD and INVA primer pair, 0·25 μmol l^{-1} each STM4057 primer pair, 2·0 μl template DNA, and 0·4 U of TaKaRa Ex Taq^{TM} Hot Start Version (Takara Bio). The volume was adjusted with

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sterile distilled water to give 20 μl. PCR reaction was carried out in a Program Temperature Control System PC-701 (ASTEC Co., Ltd, Fukuoka, Japan) under the following conditions: denaturation at 95°C for 10 min, followed by 40 cycles of amplification (denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min), ending with a final extension at 72°C for 15 min. The amplified products were separated by electrophoresis on 2·5% agarose gels in 1 × Tris-acetate-EDTA buffer (Wako Pure Chemical Industries Ltd, Osaka, Japan) using a Mini-Gel Electrophoresis system (Mupid®-2plus, Advance Co. Ltd, Tokyo, Japan), stained with ethidium bromide, visualized under UV irradiation and photographed with a 3UV™ Transilluminator NLMS-20E (Atto Corp., Tokyo, Japan).

Results

Specificity of each primer

The results of each monoplex PCR assay for all strains used in the present study are shown in Table 1. Detection of the *mdcA*, *gatD* and *fljB* genes correlated well with the phenotypic traits of malonate utilization, acid production from galactitol or phase 2 flagellin expression in each strain, respectively (data not shown). However, there were some discrepancies between the phenotype and genotype in some strains. The primer pair mdcA 7–8 did not amplify any product from *S. enterica* subsp. II R21D2 and R52D1. Detection of the *stn* and STM4057 gene was consistent with previous studies in most *Salmonella* strains. However, the primer pair STM4057 f1–r1 amplified the expected size product from strain *S. enterica* subsp. II Kn127M1 and primer pair stn f1–r1 did not amplify any product from *S. enterica* subsp. II R21D2. The primer pair INVA1-2 specifically amplified the expected size product from all *Salmonella* strains and no product from non-*Salmonella* strains.

Multiplex PCR

Typical examples of the multiplex PCR assay in all *Salmonella* subspecies are shown in Fig. 1. All amplified products were well separated by electrophoresis on 2·5% agarose gel. The results of the multiplex PCR assay were completely correlated with the result of each monoplex PCR in all strains used in the present study (Table 1). Boiling-lysis supernatants of each strain generated the same band patterns as template DNA extracted by the DNA purification kit (data not shown). Of the 53 *Salmonella* strains tested, 50 strains (94·3%) successfully demonstrated the unique band pattern to their subspecies according to the expected band patterns (Tables 1 and 2).

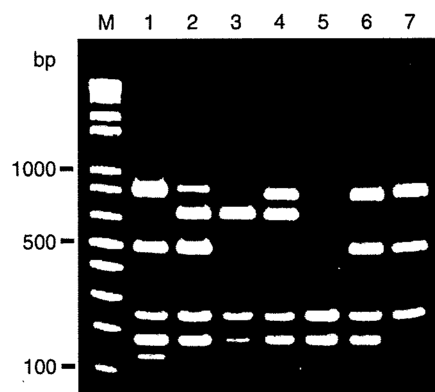

Figure 1 Agarose gel electrophoresis results for the multipler PCR assay with representative isolates of *Salmonella* subspecies. Lane 1, *S. enterica* subsp. I (NMJS1); Lane 2, *S. enterica* subsp. II (R42D2); lane 3, *S. enterica* subsp. IIIa (8M1); lane 4, *S. enterica* subsp. IIIb (WC1D1); lane 5, *S. enterica* subsp. IV (7D1); lane 6, *S. enterica* subsp. VI (CCUG30038); lane 7, *S. bongori*: (321E); lane M, 1 kb plus DNA ladder (Invitrogen).

Discussion

The multiplex PCR assay described here was found to be rapid, reliable and easy to perform, and had high ability of subspecies identification (94·3%) in 53 *Salmonella* strains (Table 1). This method can be an alternative or complementary method to traditional biochemical-based identification of *Salmonella* subspecies. Such a practical identification method of *Salmonella* at subspecies level other than biochemical tests has not been published at the time of this writing. Conventional biochemical-based identification usually requires more than 1 day and several media, and the techniques should be standardized to avoid subjective observations (Rosselló-Mora and Amann 2001). To improve the procedure, molecular biological techniques are useful, since multilocus enzyme electrophoresis, sequence analyses of chromosomal genes, and DNA microarray analysis revealed *Salmonella* subspecies are genetically distinct from each other (Boyd *et al.* 1996; Porwollik *et al.* 2002; Chan *et al.* 2003). Moreover, as the primer pair for *invA* allows simultaneous identification of *Salmonella* at the genus level, the assay is useful especially for rapid screening of large numbers of samples.

Three *Salmonella* strains were not successfully identified as their phenotypically defined subspecies. *Salmonella enterica* subsp. II R21D2 and R52D1 were identified as *S. bongori* and *S. enterica* subsp. VI, respectively. *Salmonella enterica* subsp. II Kn127M1 could not be classified into any subspecies according to the expected band patterns (Table 2). These discrepancies were responsible for variation of three genes; *mdcA*, *stn* and STM4057. In two *mdcA*-negative strains, *S. enterica* subsp. II R21D2

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and R52D1, it took 2 days to utilize malonate, while only 1 day in the other malonate positive strains. Furthermore, various *loci* of *mdcA* and other genes in the gene cluster of malonate decarboxylase (MdCa) (Hoenke *et al.* 1997) were not detected from these strains (data not shown).

Thus, it should be suggested that modification or variation exists in the gene cluster of MdCa of these two strains which utilize malonate slowly. Additional research would be needed to reveal how much this gene cluster contributes to the malonate metabolism in *Salmonella*. The distribution of the *stn* and STM4057 genes have been reported to correlate to *Salmonella* lineage (Prager *et al.* 1995; Kim H. *et al.* 2006). Thus, the cause of the discrepancies of these genes remains unclear, but may be explained by genetic variation among subspecies. In addition, it is interesting that only strains of *S. enterica* subsp. II were not successfully identified. Our data suggest that this subspecies might be a genetically heterogeneous group. Although a large amount of genetic information is required to verify this hypothesis, whole genome sequence information of *S. enterica* subsp. II, IV and VI is not available at the time of this writing. Further genetic studies on these subspecies may provide a novel insight to their phylogeny or subspecies-specific regions.

For more accurate identification, we recommend additional one or two biochemical tests when sample strains demonstrate the unique band patterns of *S. enterica* subsp. VI or *S. bongori*, or untypable patterns by the assay. It only requires one or two kinds of tests that discriminate between *S. enterica* subsp. II and *S. enterica* subsp. VI or *S. bongori* (e.g. the utilization of malonate, acid production from galactitol or the use of commercial identification kits). It is less labour-intensive than identification only by several biochemical tests. Moreover, since *S. enterica* subsp. VI and *S. bongori* are rarely isolated even from reptiles, from which other uncommon *Salmonella* subspecies are often isolated (Geue and Loschner 2002; Pasmans *et al.* 2005), such case will not frequently occur in field investigations.

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References

Anon (2004) Cultural methods. In Collins, Lyne's Microbiological Methods ed. Collins, C.H., Lyne, P.M., Grange, J.M. and Falkinhamm, J.O. III pp. 81–88. London: Hodder Arnold.

Bäumler, A.J. and Heffron, F. (1998) Structure of the *smpB-nrdE* intergenic region of *Salmonella enterica*. *J Bacteriol* **180**, 2220–2223.

Bäumler, A.J., Hargis, B.M. and Tsolis, R.M. (2000) Enhanced: tracing the origins of *Salmonella* outbreaks. *Science* **287**, 50–52.

Bopp, C.A., Brenner, F.W., Fields, P.I., Wells, J.G. and Strockbine, N.A. (2003) *Escherichia*, *Shigella*, and *Salmonella*. In: *Manual of Clinical Microbiology* ed. Murray, P.R., Baro, E.J., Jorgensen, J.H., Pfaller, M.A. and Yolken, R.H. pp. 654–671. Washington, DC: ASM Press.

Boyd, E.F., Wang, F., Whittam, T.S. and Selander, R.K. (1996) Molecular genetic relationships of the Salmonellae. *Appl Environ Microbiol* **62**, 804–808.

Centers for Disease Control and Prevention (2003) Reptile-associated Salmonellosis – selected states, 1998–2002. *MMWR Morb Mortal Wkly Rep* **52**, 1206–1209.

Chan, K., Baker, S., Kim, C.C., Detweiler, C.S., Dougan, G. and Falkow, S. (2003) Genomic comparison of *Salmonella enterica* serovars and *Salmonella bongori* by use of an *S. enterica* serovar Typhimurium DNA microarray. *J Bacteriol* **185**, 553–563.

Chiu, C.H. and Ou, J.T. (1996) Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, *invA* and *spvC*, by an enrichment broth culture-multiplex PCR combination assay. *J Clin Microbiol* **34**, 2619–2622.

D'Aoust, J-Y. and Maurer, J. (2007) *Salmonella* Species. In *Food Microbiology* ed. Doyle, M.P. and Beuchat, L.R. pp. 187–236. Washington DC: ASM Press.

De Jong, B., Anderson, Y. and Ekdahl, K. (2005) Effect of regulation and education on reptile-associated Salmonellosis. *Emerg Infect Dis* **11**, 398–403.

Geue, L. and Loschner, U. (2002) *Salmonella enterica* in reptiles of German and Austrian origin. *Vet Microbiol* **84**, 79–91.

Giammanco, G.M., Pignato, S., Mammina, C., Grimont, F., Grimont, P.A.D., Nastasi, A. and Giammanco, G. (2002) Persistent endemicity of *Salmonella bongori* 48:z35:- in southern Italy: Molecular characterization of human, animal, and environmental isolates. *J Clin Microbiol* **40**, 3502–3505.

Heinzerling, A.F. and Bockemuhl, J. (1996) Human infection caused by Salmonellae of subspecies II to VI in Germany, 1977–1992. *Zentralbl Bakteriol* **283**, 391–398.

Herrera-León, S., McQuiston, J.R., Usera, M.A., Fields, P.I., Garaizar, J. and Echeita, M.A. (2004) Multiplex PCR for distinguishing the most common phase-1 flagellar antigens of *Salmonella* spp. *J Clin Microbiol* **42**, 2581–2586.

Hoenke, S., Schmid, M. and Dimroth, P. (1997) Sequence of a gene cluster from *Klebsiella pneumoniae* encoding malonate decarboxylase and expression of the enzyme in *Escherichia coli*. *Eur J Biochem* **246**, 530–538.

Kaibu, H., Iida, K., Ueki, S., Ehara, H., Simasaki, Y., Anzai, H., Toku, Y. and Shirono, S. (2006) Salmonellosis of

© 2009 The Authors

infants presumably originating from an infected turtle in Nagasaki, Japan. *Jpn J Infect Dis* **59**, 281.

Kim, S., Frye, J.G., Hu, J., Fedorka-Cray, P.J., Gautom, R. and Boyle, D.S. (2006) Multiplex PCR-based method for identification of common clinical serotypes of *Salmonella enterica* subsp. *enterica*. *J Clin Microbiol* **44**, 3608–3615.

Kim, H., Park, S. and Kim, H. (2006) Comparison of *Salmonella enterica* serovar Typhimurium LT2 and non-LT2 *Salmonella* genomic sequences, and genotyping of Salmonellae by using PCR. *Appl Environ Microbiol* **72**, 6142–6151.

Malorny, B., Hoorfar, J., Bunge, C. and Helmuth, R. (2003) Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. *Appl Environ Microbiol* **69**, 290–296.

Miyamoto, T., Trevanich, S., Honjoh, K. and Hatano, S. (1999) Rapid detection of *Salmonella* spp. by PCR amplification of *Salmonella* specific region in *gatD* gene. *Jpn J Food Microbiol* **16**, 99–109.

Moore, M.M. and Feist, M.D. (2007) Real-time PCR method for *Salmonella* spp. targeting the *stn* gene. *J Appl Microbiol* **102**, 516–530.

Nakadai, A., Kuroki, T., Kato, Y., Suzuki, R., Yamai, S., Yaginuma, C., Shiotani, R., Yamanouchi, A. *et al.* (2005) Prevalence of *Salmonella* spp. in pet reptiles in Japan. *J Vet Med Sci* **67**, 97–101.

O'Hara, C.M. (2005) Manual and automated instrumentation for identification of *Enterobacteriaceae* and other aerobic gram-negative bacilli. *Clin Microbiol Rev* **18**, 147–162.

Pasmans, F., Martel, A., Boyen, F., Vandekerchove, D., Wybo, I., Immerseel, F.V., Heyndrickx, M., Collard, J.M. *et al.* (2005) Characterization of *Salmonella* isolates from captive lizards. *Vet Microbiol* **110**, 285–291.

Popoff, M.Y. (2001) *Antigenic Formulas of the Salmonella Serovars*, 8th edn. Paris, France: WHO Collaborating Centre for Reference and Research on Salmonella, Institute Pasteur.

Popoff, M.Y. and Le Minor, L.E. (2005) Genus XXXIII. *Salmonella*. in *Bergey's Manual of Systematic Bacteriology* ed. Brenner, D.J., Krieg, N.R. and Staley, J.T. pp. 764–799. Baltimore, MD: Williams and Wilkins.

Porwollik, S., Wong, R.M. and McClelland, M. (2002) Evolutionary genomics of *Salmonella*: gene acquisitions revealed by microarray analysis. *Proc Natl Acad Sci U S A* **99**, 8956–8961.

Prager, R., Fruth, A. and Tschäpe, H. (1995) *Salmonella* enterotoxin (*stn*) gene is prevalent among strains of *Salmonella enterica*, but not among *Salmonella bongori* and other Enterobacteriaceae. *FEMS Immunol Med Microbiol* **12**, 47–50.

Römkens, T.E.H., Hekker, T.A.M. and Smulders, Y.M. (2003) Turtle-associated human Salmonellosis. *Clin Infect Dis* **37**, e167–e169.

Rosselló-Mora, R. and Amann, R. (2001) The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39–67.

Sanyal, D., Douglas, T. and Roberts, R. (1997) *Salmonella* infection acquired from reptilian pets. *Arch Dis Child* **77**, 345–346.

Shelobolina, E.S., Sullivan, S.A., O'Neil, K.R., Nervin, K.P. and Lovely, D.R. (2004) Isolation, characterization, and U(VI)-reducing potential of a facultatively anaerobic, acid-resistant bacterium from low-pH, nitrate- and U(VI)-contaminated subsurface sediment and description of *Salmonella subterranea* sp. nov. *Appl Environ Microbiol* **70**, 2959–2965.

Tavechio, A.T., Ghilardi, Â.C.R., Peresi, J.T.M., Fuzihara, T.O., Yonamine, E.K., Jakabi, M. and Fernandes, S.A. (2002) *Salmonella* serotypes isolated from nonhuman sources in São Paulo, Brazil, from 1996 through 2000. *J Food Prot* **65**, 1041–1044.

Tindall, B.J., Grimont, P.A.D., Garrity, G.M. and Euzéby, J.P. (2005) Nomenclature and taxonomy of the genus *Salmonella*. *Int J Syst Evol Microbiol* **55**, 521–524.

Woodward, D.L., Khakhria, R. and Johnson, W.M. (1997) Human salmonellosis associated with exotic pets. *J Clin Microbiol* **35**, 2786–2790.

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NOTE *Pathology*

Spontaneous Yersiniosis Due to *Yersinia pseudotuberculosis* Serotype 7 in a Squirrel Monkey

Shin-ichi NAKAMURA[1)], Hideki HAYASHIDANI[2)], Taketoshi IWATA[2)], Mariko TAKADA[3)] and Yumi UNE[1)]*

[1)]*Laboratory of Veterinary Pathology, Azabu University, 1–17–71 Fuchinobe, Sagamihara, Kanagawa 229–8501 and* [2)]*Institute of Symbiotic Science and Technology, Tokyo University of Agriculture and Technology, 3–5–8 Saiwai-cho, Fuchu, Tokyo 183–8509 and* [3)]*Uminonakamichi Seaside Park Administration, 18–25 Oaza Saitozaki, Higashi-ku, Fukuoka 811–0321, Japan*

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ABSTRACT. A captive male Bolivian squirrel monkey (*Saimiri boliviensis*) of less than 1 year of age died following diarrhea and debilitation on the day of death. At necropsy, necrotizing enteritis accompanied with enlarged Peyer's patches, solitary lymphatic follicles and mesenteric lymph nodes, and multiple yellowish-white nodules in the spleen and liver were found. Histopathologically, these lesions were necrotizing inflammation containing Gram-negative bacilli. *Yersinia pseudotuberculosis* serotype 7 was isolated from the spleen and liver. The *virF* gene, which is an essential virulent plasimid (pYV) in pathogenic *Y. pseudotuberculosis* isolates, and the *ypmA* gene, which is a superantigenic toxin, were detected in the isolates. This is the first report of a fatal case of *Yersinia pseudotuberculosis* 7 infection in the world.

KEY WORDS: pathogenic Yersinia, squirrel monkey, *Yersinia pseudotuberculosis* 7 infection.

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Yersiniosis refers to infections caused by either *Yersinia enterocolitica* or *Yersinia pseudotuberculosis*, which appear as enteritis and sometimes as septicemia in humans and animals [12]. *Y. pseudotuberculosis* is detected in many animal species including wild animals, livestock, and companion animals. Many cases of yersiniosis have been reported in Japan, especially in nonhuman primates with high sensibility to *Y. pseudotuberculosis* in zoos [8, 10, 19]. This trend is also seen overseas [2, 11, 15, 17]. *Y. pseudotuberculosis* is a Gram-negative bacillus and it has been classified into serotypes O:1 to O:15 based on O-antigen, and 7 pathogenic serotypes, i.e., 1 to 6 and 10. Other serotypes including 7 are derived from the environment, and are known to be non-pathogenic [7, 14]. Most of the strains from Europe belong to serotypes 1a and 3 [18]. In contrast, *Y. pseudotuberculosis* strains belonging to three serotypes (4b, 5a, and 5b) have been isolated from human patients in Japan, and 5 serotypes (1b, 2b, 3, 4b, and 6) have been influenced by animals [3, 5, 16, 18]. The pathogenicity of *Y. pseudotuberculosis* is associated with several virulence factors. Pathogenic strains of *Y. pseudotuberculosis* have a 70-kb virulence plasimid (pYV). Additionally, a high-pathogenicity island (HPI), *Y. pseudotuberculosis*-derived mitogen (YPM), which is a superantigenic toxin, and invasin, which allows efficient entry into mammalian cells, are known to play important roles in causing severe systemic infection [9].

In this study, we report the first instance ever of a fatal case of *Yersinia pseudotuberculosis* 7 infection.

In June 2003, a captive male Bolivian squirrel monkey (*Saimiri boliviensis*) of less than 1 year of age was found moribund and soon died. The monkey had diarrhea (mucous and bloody stool) on the day of death, and its weight was 380 g. It was the only such case to occur, and was subjected to necropsy. For histopathological examination, specimens of various tissues and organs were fixed in neutral buffered 10% formalin and embedded in paraffin wax. Sections (approximately 3 μm) were cut and stained with hematoxylin-eosin (HE) and Gram stain (Brown-Hopps method). Immunohistochemical examination was done using a commercial rabbit anti-*Y. pseudotuberculisis* 1–6 sera (Denka-Seiken, Co., Tokyo, Japan) and an indirect method. Bacteriological examination of the spleen and liver was also done.

At necropsy, there were bloody ascites, swelling of Peyer's patches, mesenteric lymph nodes, and enlargement of the spleen and liver with multi-focal yellowish-white nodules. Pseudomembranous enterocolitis was associated with the Peyer's patches and solitary lymphatic nodules and mucosa of the small intestine, and these were sometimes accompanied by hemorrhages. No other irregularities were seen in the lung, kidney, or heart.

Histopathologically, nodules seen in the liver and spleen were foci of necrosis accompanied by infiltration of neutrophils and macrophages containing Gram-negative bacilli (Figs. 1, 2, and 3). Lesions in both the small and large intestine were characterized by small and large foci of necrosis with ulceration and erosion of the mucosa, mainly Peyer's patches and solitary lymphatic nodules (Fig. 4). Occasionally the lesions extended to the submucosa. There was also desquamation of the mucosal epithelium and congestion, haemorrhage and accumulations of nuclear debris and numerous bacterial colonies in the lesions. Severe neutrophil infiltration was apparent. The mesenteric lymph nodes were markedly expanded by the influx of edema fluid and by large numbers of neutrophils and macrophages mixed with bacterial colonies. Necrotic foci with neutrophils and

* CORRESPONDENCE TO: UNE, Y., Laboratory of Veterinary Pathology, School of Veterinary Medicine, Azabu University, 1–17–71 Fuchinobe Sagamihara, Kanagawa 229–8501, Japan.
e-mail: une@azabu-u.ac.jp

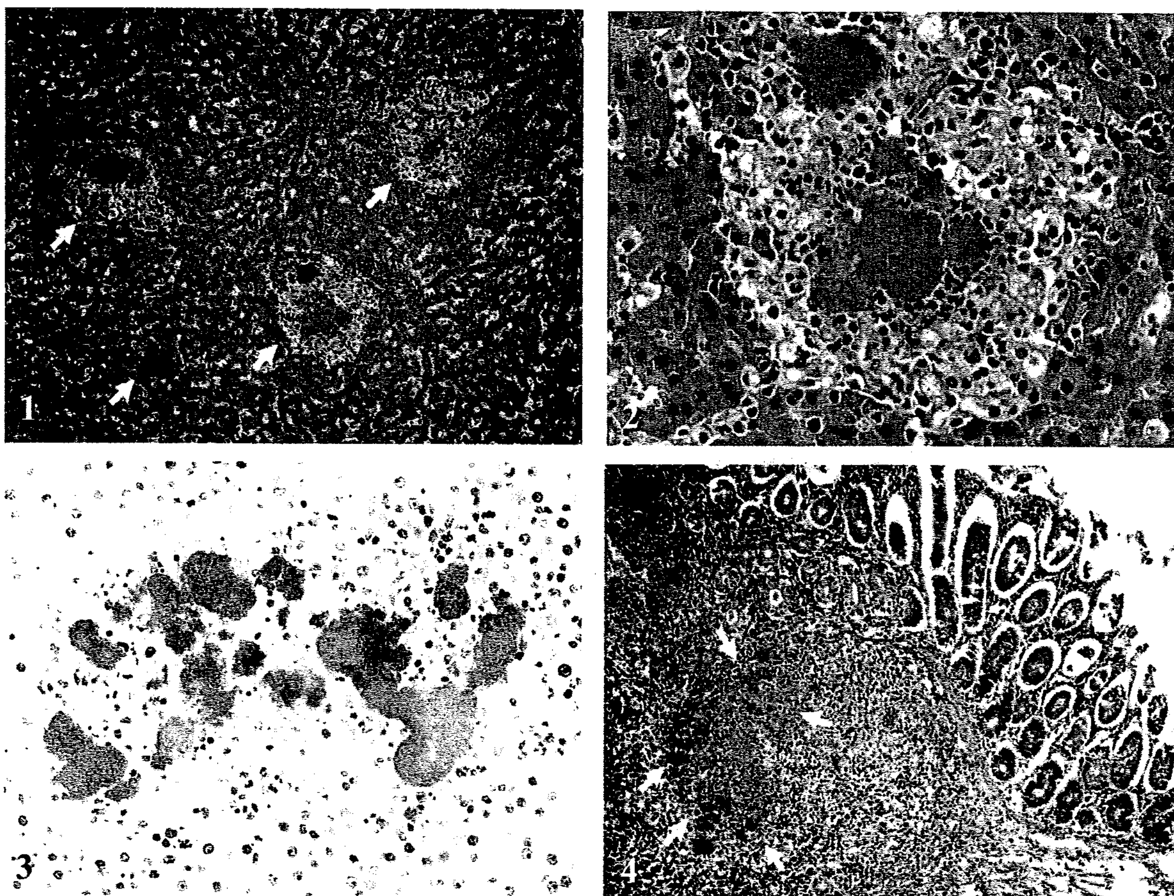

Fig. 1. Liver. Multi-focal necrosis accompanied by infiltration of neutrophils and macrophages included numerous bacterial colonies (arrows). Hematoxylin and Eosin (HE). × 100

Fig. 2. Higher magnification of necrotizing foci in the liver. HE. × 400

Fig. 3. Liver. Bacterial masses in the multi-focal necrosis were Gram-negative bacilli. Gram stain. × 200

Fig. 4. Peyer's patch. A lymphoid follicle of Peyer's patch replaced by numerous bacterial colonies and inflammatory cells (arrows). HE. × 100

lymphocytes were seen in the kidneys, but there were no bacterial colonies. Other findings included swelling and vacuolar degeneration of hepatocytes, myocardial degeneration, and pulmonary edema. Bacterial colonies seen in lesions were immunohistochemically negative to slightly positive for anti-*Y. pseudotuberculosis* 1–6 sera.

Only *Y. pseudotuberculosis* 7 was isolated from the spleen and liver. Microbiological features of isolates were described in a previous report [9]. Briefly, serotyping of isolates was performed by slide agglutination with rabbit immune sera and polymerase chain reaction (PCR) as described by Bogdanovich *et al.* (2003) [1]. The presence of the virulence genes *virF*, *inv*, *ypm* (*ypmA*, *ypmB*, and *ypmC*) and *irp2* were confirmed by PCR. The *virF* and *irp2* genes were used as markers for the presence of pYV and HPI, respectively. An isolated strain had *virF*, *inv* and *ypmA*, but not *ypmB*, *ypmC*, or *irp2*.

This animal had localized necrotizing enteritis in the lymphoid organs and enlarged spleen and liver accompanied with multi-focal necrosis with intralesional Gram-negative bacilli. These lesions were typical of those seen in other squirrel monkeys infected with *Y. pseudotuberculosis*. A diagnosis of yersiniosis was confirmed by isolation of *Y. pseudotuberculosis* in pure culture and PCR from the spleen and liver. On the basis of these findings, this case was diagnosed as yersiniosis due to *Yersinia pseudotuberculosis* serotype 7, and we concluded that the monkey died of sepsis caused by *Yersinia pseudotuberculosis* serotype 7.

To our knowledge, this is the first reported fatal case due to *Y. pseudotuberculosis* serotype 7 infection. This serotype has been isolated from healthy dogs, moles, and wild mice, but did not have pathogenic plasmids, and it was thought to be a non-pathogenic strain [4, 6, 13]. The PCR analysis demonstrated that this strain also had pYV and *ypmA* genes.

The pathological findings of this case were not different from those of other serotypes. Also, this case did not show immunological deterioration or ateliosis. These results suggest that the strain serotype 7 isolated in the present study has the same degree of pathogenicity as other pathogenic serotypes.

In Japan, *Y. pseudotuberculosis* has been isolated from human patients and from various animals including wild animals, livestock, and companion animals, and various serotypes have been isolated. Because there have been no reports about *Y. pseudotuberculosis* serotype 7 isolated from human patients and animals, additional pathological and epidemiological studies are necessary, and we should pay attention to the possibility of fatal infection in humans and other animals by serotype 7.

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REFERENCES

1. Bogdanovich, T., Carniel, E., Fukushima, H. and Skurnik, M. 2003. Use of O-antigen gene cluster-specific PCRs for the identification and O-genotyping of *Yersinia pseudotuberculosis* and *Yersinia pestis*. *J. Clin. Microbiol.* **41**: 5103–5112.
2. Buhles, W. C. Jr., Vanderlip, J. E., Russell, S. W. and Alexander, N. L. 1981. *Yersinia pseudotuberculosis* infection: study of an epizootic in squirrel monkeys. *J. Clin. Microbiol.* **13**: 519–525.
3. Fukushima, H. 2000. *Yersinia pseudotuberculosis*. pp. 321–335. *In*: Food and Water-Borne Infection and Food Poisoning (Sakazaki, R. ed.), Chuohoki, Tokyo(in Japanese).
4. Fukushima, H., Gomyoda, M. and Kaneko, S. 1990. Mice and moles inhabiting mountainous areas of Shimane Peninsula as sources of infection with *Yersinia pseudotuberculosis*. *J. Clin. Microbiol.* **28**: 2448–2455.
5. Fukushima, H., Maruyama, T., Kaneko, K. and Inoue, M. 1989. Yersiniosis and the ecology of *Yersinia*. *J. Jpn. Vet. Med. Assoc.* **42**: 829–840(in Japanese).
6. Fukushima, H., Matsuda, Y., Seki, R., Tsubokura, M., Takeda, N., Shubin, F. N., Paik, I. K. and Zheng, X. B. 2001. Geographical heterogeneity between Far Eastern and Western countries in prevalence of the virulence plasmid, the superantigen *Yersinia pseudotuberculosis*-derived mitogen, and the high-pathogenicity island among *Yersinia pseudotuberculosis strains*. *J. Clin. Microbiol.* **39**: 3541–3547.
7. Hayashidani, H. 2005. *Yersinia pseudotuberculosis* infection. *Modern Media.* **51**: 211–216.
8. Hirai, K. 1974. *Yersinia pseudotuberculosis* infection occurred spontaneously in a group of Patas monkeys (*Erythrocebus patas*). *Jpn. J. Vet. Sci.* **36**: 351–355.
9. Iwata, T., Une, Y., Okatani, A. T., Kato, Y., Nakadai, A., Lee, K., Watanabe, M., Taniguchi, T., Elhelaly, A. E., Hirota, Y. and Hayashidani, H. 2008. Virulence characteristics of *Yersinia pseudotuberculosis* isolated from breeding monkeys in Japan. *Vet. Microbiol.* **129**: 404–409.
10. Kageyama, T., Ogasawara, A., Fukuhara, R., Narita, Y., Miwa, N., Kamanaka, Y., Abe, M., Kumazaki, K., Maeda, N., Suzuki, J., Gotoh, S., Matsubayashi, K., Hashimoto, C., Kato, A. and Matsubayashi, N. 2002. *Yersinia pseudotuberculosis* infection in breeding monkeys:detection and analysis of strain diversity by PCR. *J. Med. Primatol.* **31**: 129–135.
11. MacArthur, J. A. and Wood, M. 1983. Yersinosis in a breeding unit of *Macaca fascicularis* (cynomolgus monkeys). *Lab. Anim.* **17**: 151–155.
12. Mair, N. S. 1973. Yersiniosis in wildlife and its public health implications. *J. Wildl. Dis.* **9**: 64–71.
13. Maruyama, T. 1982. Yersiniosis as s zoonosis. *J. Jpn. Vet. Med. Assoc.* **35**: 2–8 (in Japanese).
14. Nagano, T. and Tsubokura, M. 1996. Serogroups of *Yersinia pseudotuberculosis* and related some problems. *J. Jpn. Vet. Med. Assoc.* **49**: 509–515 (in Japanese).
15. Rosenberg, D. P., Lerche, N. W. and Henrickson, R. V. 1980. *Yersinia pseudotuberculosis* in a group of *Macaca fascicularis*. *J. Am. Vet. Med. Assoc.* **177**: 818–819.
16. Sasaki, A., Shimada, A., Awakura, T., Umemura, T., Nagano, T., Sanekata, T. and Tsubokura, M. 1996. Pathology of spontaneous infection with *Yersinia pseudotuberculosis* in a common squirrel monkey. *J. Jpn. Vet. Med. Assoc.* **149**: 819–821 (in Japanese).
17. Taffs, L. F. and Dunn, G. 1983. An outbreak of *Yersinia pseudotuberculosis* infection in a small indoor breeding colony of red-bellied (*Saguinus labiatus*) tamarins. *Lab. Anim.* **17**: 311–320.
18. Tsubokura, M. 1987. *Yersinia pseudotuberculosis*. *J. Jpn. Vet. Med. Assoc.* **40**: 317–323 (in Japanese).
19. Une, Y., Isobe, K., Baba, T., Hayashidani, H. and Nomura, Y. 2003. *Yersinia pseudotuberculosis* in squirrel monkeys. *Jpn. J. Zoo. Wildl. Med.* **8**: 19–26 (in Japanese).

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Loop-Mediated Isothermal Amplification-Based Diagnostic Assay for Monkeypox Virus Infections

Itoe Iizuka,[1,2] Masayuki Saijo,[1]* Tomoyuki Shiota,[1,2] Yasushi Ami,[3] Yuriko Suzaki,[3] Noriyo Nagata,[4] Hideki Hasegawa,[4] Kouji Sakai,[1,3] Shuetsu Fukushi,[1] Tetsuya Mizutani,[1] Momoko Ogata,[1] Mina Nakauchi,[1] Ichiro Kurane,[1] Masashi Mizuguchi,[2] and Shigeru Morikawa[1]

[1]*Department of Virology 1, National Institute of Infectious Diseases, Musashimurayama, Tokyo, Japan*
[2]*Faculty of Medicine, Department of International Health, The University of Tokyo, Bunkyo-ku, Tokyo, Japan*
[3]*Department of Experimental Animals, National Institute of Infectious Diseases, Musashimurayama, Tokyo, Japan*
[4]*Department of Pathology, National Institute of Infectious Diseases, Musashimurayama, Tokyo, Japan*

Monkeypox virus (MPXV) causes a smallpox-like disease in non-human primates and humans. This infection is endemic to central and western Africa. MPXV is divided into two genetically different groups, Congo Basin and West African MPXV, with the former being the more virulent. A real-time quantitative MPXV genome amplification system was developed for the diagnosis of MPXV infections using loop-mediated isothermal amplification (LAMP) technology. Primers used for genome amplification of Congo Basin (C-LAMP), West African (W-LAMP), and both Congo Basin and West African (COM-LAMP) MPXV by LAMP were designed according to the nucleotide sequences of the Congo Basin-specific D14L gene, the West African-specific partial ATI gene, and the partial ATI gene that is shared by both groups, respectively. The sensitivity and specificity of the LAMP were evaluated with nested PCR using peripheral blood and throat swab specimens collected from Congo Basin MPXV or West African MPXV-infected monkeys. The sensitivity and specificity of COM-LAMP, C-LAMP, and W-LAMP were 80% (45/56) and 100% (64/64); 79% (19/24) and 100% (24/24); and 72% (23/32) and 100% (40/40), respectively. The viremia level determined by LAMP assays increased with increases in the severity of the monkeypox-associated symptoms. The newly developed LAMP assay was confirmed to be a rapid, quantifiable, and highly sensitive and specific system effective in the diagnosis of MPXV infections. The LAMP assays made it possible to discriminate between Congo Basin and West African MPXV. The LAMP developed in this study is useful not only for diagnosis of but also for the assessment of MPXV infections. ***J. Med. Virol. 81:1102–1108, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: loop-mediated isothermal amplification; LAMP; monkeypox; monkeypox virus; diagnosis

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INTRODUCTION

Monkeypox virus (MPXV) belongs to the genus *orthopoxvirus* in family *Poxviridae*, and, together with variola virus, is a causative agent for smallpox. MPXV was first isolated in 1958 from cynomolgus monkeys with symptoms similar to those of smallpox in humans [von Magnus et al., 1959]. It was identified that MPXV causes a smallpox-like disease in humans (human monkeypox) in 1970 [Ladnyj et al., 1972]. The disease is endemic to the rainforests of central and western Africa, where some species of ground squirrels, which are suggested to be the reservoir, are prevalent [Meyer et al., 2002]. People living in the endemic regions still suffer from this infection. A sporadic outbreak of human monkeypox occurred in the USA in 2003 [Likos et al., 2005]. This was the first outbreak of human monkeypox outside Africa. The source of the outbreak was MPXV-infected ground squirrels, such as African dormice and Gambian giant rats, imported from western Africa through Accra, Ghana. This event indicates that there is the possibility that MPXV could spread outside Africa and cause human monkeypox even in countries currently free from this infection. Furthermore, MPXV is categorized as an important bio-weapons. MPXV is divided into two genetic distinct groups, Congo Basin and West African MPXV. Congo Basin MPXV was

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*Correspondence to: Masayuki Saijo, MD, PhD, Department of Virology 1, National Institute of Infectious Disease, Tokyo 208-0011, Japan. E-mail: msaijo@nih.go.jp

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reported to be more virulent than West African MPXV [Chen et al., 2005]. Therefore, there is a need to develop rapid and accurate diagnostic systems with which to discriminate between Congo Basin and West African MPXV.

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method that works by autocycling strand displacement DNA synthesis by *Bst* DNA polymerase, and has been confirmed to be simple to use [Notomi et al., 2000]. The amplification is conducted under isothermal conditions ranging from 60 to 65°C with DNA polymerase and, usually, four primers recognizing six distinct target regions (4-primer-based LAMP), making this assay highly specific. If two additional "loop primers" are included in the LAMP assay, the reaction time can be reduced (6-primer-based LAMP) [Nagamine et al., 2002]. Recently, the LAMP-based diagnosis for several virus infections have been developed [Hong et al., 2004; Kaneko et al., 2005; Imai et al., 2006; Parida et al., 2006, 2007; Suzuki et al., 2006; Shirato et al., 2007].

In the present study, 6-primer-based LAMP was applied for the development of diagnostic systems for MPXV infections. Three LAMP assays, which detected specifically Congo Basin and West African MPXV, Congo Basin but not West African MPXV, and West African but not Congo Basin MPXV, respectively, were developed. The usefulness of the LAMP assays for the diagnosis and assessment of MPXV infections was evaluated using peripheral blood and throat swab specimens collected from MPXV-infected monkeys.

MATERIALS AND METHODS

Viruses and Cells

Congo Basin MPXV (Zr-599, Congo-8, and V97-I-008 strains), West African MPXV (Sierra Leone, Liberia, Copenhagen, and Anteatan strains), cowpox virus (Brighton Red strain), camelpox virus (J1 strain), ectromelia virus (Hamstead strain), and vaccinia virus (Lister strain) stored at the National Institute of Infectious Diseases, Tokyo, Japan (NIID), were used. The MPXV strains and other orthopoxviruses were grown on Vero and HeLa cells, respectively.

Extraction and Purification of Virus Genome

Viral DNA was extracted from MPXV (Zr-599)-, MPXV (Liberia)-, cowpox virus-, camelpox virus-, ectromelia virus-, or vaccinia virus-infected cells using the Hirt extraction method [Hirt, 1967]. Viral DNA in the peripheral blood and throat swab specimens was purified using a Viral Nucleic Acid Purification Kit™ (Roche Diagnostics Ltd., Rotkreuz, Switzerland) and stored at −30°C until use.

Real-Time Quantitative PCR for Orthopoxvirus

A SYBR Green-based real-time quantitative PCR (pox-qPCR) was developed. Primers, forward primer [H2Rf (5′-CGGTTAACGATTGGAAATCATTAACGG-3′)] and reverse primer [H2Rr (5′-CCTCGCCTAA-TAGCTTGCG-3′)], used in the pox-qPCR were designed according to the nucleotide sequences of the *H2R* gene shared by viruses in the genus *Orthopoxvirus*. Standard DNA, a pGEM-T easy vector (GH Health Care Japan, Tokyo, Japan) inserted with the partial *H2R* gene amplified in PCR with the above primer set (H2f and H2r), was used for the determination of the copy number of the virus genome of MPXV, cowpox virus, camelpox virus, ectromelia virus, and vaccinia virus. The reaction conditions were as follow: one cycle of 95°C for 10 min for denaturation, 45 cycles of 95°C for 15 sec, 63°C for 5 sec, 72°C for 10 sec, followed by one cycle of 73°C for 15 sec. PCR amplification was performed using a LightCycler FastStart DNA Master SYBR Green I™ kit (Roche Diagnostics Ltd.) in a 20 µl-volume format containing 5 µl of template DNA according to the manufacturer's instructions.

LAMP

The 6-primer-based LAMP consists of six primers; two outer primers (F3 and B3), a forward inner primer (FIP), a backward inner primer (BIP), and two loop primers (LF and LB) [Nagamine et al., 2002]. Three LAMP assays were developed for the amplification of the genomes of both Congo Basin and West African MPXV (COM-LAMP), of the genomes of Congo Basin but not West African MPXV (C-LAMP), and of the genomes of West African but not Congo Basin MPXV (W-LAMP). The primers for COM-LAMP, C-LAMP, and W-LAMP were designed according to the nucleotide sequences of the A-type inclusion body (ATI) shared by both Congo Basin and West African MPXV, those of the Congo Basin MPXV-specific *D14L* gene [Likos et al., 2005], and those of the West African MPXV-specific partial ATI gene [Saijo et al., 2008]. The primer Loop-B-COM in the COM-LAMP was designed to anneal the region containing a deletion of eight nucleotide residues observed only in MPXV but not in other orthpoxviruses [Neubauer et al., 1998]. The nucleotide sequences of the primers used in each LAMP assay are shown in Table I. The LAMP reaction was performed with a Loopamp DNA Amplification kit™ (Eiken Chemical Co., Ltd., Tochigi, Japan). The reaction mixture (25 µl) containing 40 pmol of each inner primer, FIP and BIP, 5 pmol of each outer primer, F3 and B3, and 20 pmol of each loop primer, LF and LB, 2 times concentrated reaction mix (12.5 µl), *Bst* polymerase (1.0 µl), and 2.0 µl of sample was incubated at 63°C with a Loopamp real-time turbidmeter (LA-200; Teramecs, Tokyo, Japan) for 1 hr, followed by incubation at 80°C for 2 min to terminate the reaction. To confirm whether the LAMP amplification products were authentic, they were digested with a designated restriction enzyme and electrophoresed in a 3% agarose gel containing ethidium bromide for separation. The DNA fragments were then visualized. The amplified COM-LAMP and C-LAMP products were digested with *Taq*I, and the W-LAMP product was digested with *Bgl*II.

TABLE I. Names and Sequences of Primers for COM-LAMP, C-LAMP, W-LAMP

Assay	Target gene	Primers	
		Name	Nucleotide sequence
COM-LAMP	ATI	FIP-COM	5′-TGGAGTCTGCTAATCTCTGTAAGATTAGAGAACTAGAGAATAAGTTGACC-3′
		F3-COM	5′-CACAAGAAGTTGATGCACTG -3′
		BIP-COM	5′-TGAGTGAATGCCGTGGAAATGCGCAGTCGTTCAACTGTA-3′
		B3-COM	5′-CAGCATTGATTTCATTATTACGT-3′
		Loop-F-COM	5′-CGCTCTCGATGCAGTC-3′
		Loop-B-COM	5′-CAGAGATTACAATCTAGAATCTCAG-3′
C-LAMP	D14L	FIP-C	5′-TGGGAGCATTGTAACTTATAGTTGCCCTCCTGAACACATGACA-3′
		F3-C	5′-TGGGTGGATTGGACCATT-3′
		BIP-C	5′-ATCCTCGTATCCGTTATGTCTTCCCACCTATTTGCGAATCTGTT-3′
		B3-C	5′-ATGGTATGGAATCCTGAGG-3′
		Loop-F-C	5′-GATATTCGTTGATTGGTAACTCTGG-3′
		Loop-B-C	5′-GTTGGATATAGATGGAGGTGATTGG-3′
W-LAMP	ATI	FIP-W	5′-CCGTTACCGTTTTACAATCGTTAATCAATGCTGATATGGAAAAGAGA-3′
		F3-W	5′-TACAGTTGAACGACTGCG-3′
		BIP-W	5′-ATAGGCTAAAGACTAGAATCAGGGATTCTGATTCATCCTTTGAGAAG-3′
		B3-W	5′-AGTTCAGTTTTATATGCCGAAT-3′
		Loop-F-W	5′-GATGTCTATCAAGATCCATGATTCT-3′
		Loop-B-W	5′-TCTTGAACGATCGCTAGAGA-3′

Standard DNA for Quantification in Each LAMP Assay

Standard DNA for the determination of the copy number of MPXV DNA was the pGEM-T easy vector inserted with the partial ATI gene amplified using the primer set, ATI-up-1 and ATI-low-1, for COM-LAMP and W-LAMP [Meyer et al., 1994]. The standard DNA for C-LAMP was the pGEM-T easy vector inserted with the partial *D14L* gene amplified using the primer set D14L-F (5′-GTTGTATGAGAGTATGATC-3′) and D14L-R (5′-TATGAAGGTGGAGAGCGTGAC-3′).

Nested PCR

A nested PCR, with which very low copies of the MPXV genomes could be amplified, was developed. The first round PCR was carried out using the primer set Gabon-1 (5′-GAGAGAATCTCTTGATAT-3′) and Gabon-2 (5′-ATTCTAGATTGTAATC-3′) [Neubauer et al., 1998]. The primers for the second round PCR were designed as follows: forward primer nest-Fa1 (5′-GCACACGCAATCAAGAAGAC-3′) and reverse primer nest-Ra1 (5′-ATTGTAATCTCTGTAGCATTTC-3′) to amplify the inner region of the product in the first round PCR. The reaction conditions were as follow: one cycle of 94°C for 5 min for denaturation, followed by 25 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, followed by one cycle of 72°C for 5 min. The PCR were performed using the GeneAmp PCR system 9700 (Applied Biosystems, Carlsbad, CA) with the Expand High Fidelity System™ (Roche Diagnostics Ltd.) according to the manufacturer's instructions.

Monkeys and MPXV-Challenge Experiments

Thirteen cynomolgus monkeys (*Macaca fascicularis*) born and raised at the Tsukuba Primate Center for Medical Science, National Institute of Biomedical Innovation, Tsukuba, Japan, were used. The monkeys were infected with MPXV in a highly contained laboratory, in which glove-box type safety cabinet was installed, at the NIID, Tokyo, Japan. All monkeys were infected with MPXV (Zr-599 or Liberia strain) at a dose of 10^6 plaque forming units. Smallpox vaccine, LC16 m8 and Lister, was confirmed to be effective in protecting the monkeys from generalized monkeypox [Saijo et al., 2006]. To demonstrate the various levels of the clinical manifestations of monkeypox, monkeys were immunized with LC16m8 or Lister for a designated period before MPXV challenge as shown in Table II. One monkey was infected with Zr-599 followed by immunization with LC16 m8. Blood samples and throat swab specimens were collected every 3 or 4 days after challenge. Clinical manifestations, such as changes in body weight, volume of food and water consumed, skin lesions, and the appearance of feces, were observed daily. The skin surface was observed carefully, and body temperature and weight were measured every 3–4 days while the monkeys were anesthetized.

Quantitative Real-Time PCR for MPXV

MPXV genome copies in the peripheral blood and throat swab specimens were determined by real-time quantitative PCR (qPCR) as reported previously [Saijo et al., 2006].

Statistical Analysis

Sensitivity and specificity were defined as the probability that the LAMP assay result was positive when the nested PCR showed a positive result, and as the probability that the LAMP assay result was negative when the nested PCR showed a negative result, respectively. The correlation coefficient(r) between the viremia levels detected by COM-LAMP and by qPCR was obtained using Pearson's correlation coefficient.

TABLE II. Experimental Data and Clinical Manifestations of Monkeypox for Monkey Subjects Used in This Study

ID	Virus inoculated	Route	Vaccination	Days from vaccination to challenge	No. of clinical samples tested		No. of papulovesicular lesions	Severity	Outcome
					PBC	TS			
Z-01-SC	Zr-599	SC	—	—	5	5	388	Severe	Sacrificed
Z-02-SC		SC	LC16m8	0	7	7	691	Severe	Survived
Z-03-SC		SC	LC16m8	3	6	6	286	Mild	Survived
Z-04-SC		SC	LC16m8	7	6	6	0	Asymptomatic	Survived
L-01-SC	Liberia	SC	—	—	7	7	196	Moderately severe	Survived
L-02-SC		SC	—	—	4	0	—	Severe	Sacrificed
L-03-SC		SC	—	—	7	0	29	Moderately severe	Survived
LC-04-IN		IN	—	—	8	0	10	Mild	Survived
LC-05-IN		IN	—	—	8	0	16	Mild	Survived
LC-06-IN		IN	Lister	42	8	0	0	Asymptomatic	Survived
LC-07-IN		IN	Lister	42	9	0	0	Asymptomatic	Survived
LC-08-IN		IN	Lister	42	7	0	0	Asymptomatic	Survived
LC-09-IN		IN	LC16m8	42	7	0	0	Asymptomatic	Survived

TS, throat swab specimens; PBC, peripheral blood cells; SC, subcutaneous inoculations; IN, intranasal inoculations.

RESULTS

Detection of Orthopoxviruses by COM-LAMP

At least 10^2 copies of MPXV Zr-599 and Liberia genomes were detected by the COM-LAMP assay. 10^6 copies of camelpox virus genomes, but only 10^5 copies of the virus genome, were positive by the COM-LAMP assay (Table III). However, the amplified DNA from the MPXV genomes could be differentiated from that from camelpox virus by restriction enzyme treatment (Fig. 1).

Detection of the Genomes of Congo Basin and West African MPXV by C-LAMP and W-LAMP

MPXV Zr-599 was detected by C-LAMP, but not by W-LAMP. On the other hand, MPXV Liberia was detected by W-LAMP but not by C-LAMP (Table III). All the Congo Basin MPXV genomes but not the West African MPXV genomes were detected by C-LAMP. On the other hand, all the West African MPXV genomes but not the Congo Basin MPXV genomes were detected by W-LAMP (data not shown).

Detection Limit of COM-LAMP, C-LAMP, and W-LAMP

The detection limits of COM-LAMP, C-LAMP, and W-LAMP were approximately $10^{2.0}$, $10^{2.4}$, 10^3 copies/reaction of standard DNA, respectively. These values were calculated from the results obtained from 9 independently repeated experiments.

Sensitivity and Specificity of the LAMP Assays

Detection of MPXV genomes in clinical samples, peripheral blood cells and throat swab specimens, was tested by each of the LAMP assays and nested PCR. The sensitivity and specificity of COM-LAMP, C-LAMP, and W-LAMP were 80% (45/56) and 100% (64/64); 79% (19/24) and 100% (24/24); and 70% (23/32) and 100% (40/40), respectively (Table IV).

Relationship Between Severity of Symptoms and Viremia Level as Determined by COM-LAMP

The severity of monkeypox symptoms observed during the challenge experiments is shown in Table II. The

TABLE III. The Reactivity of the Tested Orthopoxvirus DNAs in COM-LAMP and of MPXV DNAs in C-LAMP and W-LAMP

LAMP	Virus	Virus genome (copies/reaction)					
		10^1	10^2	10^3	10^4	10^5	10^6
COM-LAMP	MPXV Zr-599	−	+	+	+	+	+
	MPXV Liberia	−	+	+	+	+	+
	Ectromelia	NT	NT	NT	−	−	−
	Cowpox	NT	NT	NT	−	−	−
	Camelpox	NT	NT	NT	−	−	+
	Vaccinia	NT	NT	NT	−	−	−
C-LAMP	MPXV Zr-599	−	−	+	+	+	+
	MPXV Liberia	−	−	−	−	−	−
W-LAMP	MPXV Zr-599	−	−	−	−	−	−
	MPXV Liberia	−	+	+	+	+	+

NT, not tested.