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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ORIGINAL ARTICLE

A novel multiplex PCR assay for *Salmonella* subspecies identification

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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*

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

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

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 fljB 2	GACTCCATCCAGGCTGAAATCAC CGGCTTTGCTGGCATTGTAG	*fljB*	Phase 2 flagellin	848	Baumler and Heffron	d	d	–	+	–	+	+
mdcA 7 mdcA 8	GGATGTACTCTTCCATCCCCAGT CGTAGCGAGCATCTGGATATCTTT	*mdcA*	Putative malonate decarboxylase	728	This study	–	+	+	+	–	–	–
gatD P5 gatD P6	GGCGCCCATTATTATCCTATTAC CATTTCCCGGCTATTACAGGTAT	*gatD*	Galactitol-1-phosphate dehydrogenase	501	This study	+	+	–	–	–	d	+
stn f1 stn r1	CGATCCCTTTCCCGCTATC GGCGAATGAGACGCTTAAG	*stn*	*Salmonella* enterotoxin	179	This study	+	+	+	+	+	+	–
STM4057 f1 STM4057 r1	GGTGGCCTCGATGATTCCCG CCCACTTGTAGCGAGCGCCG	STM4057	Putative inner membrane protein	137	Kim H. *et al.* (2006)	+	–	–	–	–	–	–
INVA-1 INVA-2	ACAGTGCTCGTTTACGACCTGAAT AGACGACTGGTACTGATCGATAAT	*invA*	Invasion protein	244	Chiu and Ou	+	+	+	+	+	+	+

+, 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 rl (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 *inv*A 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™ Hot Start Version (Takara Bio). The volume was adjusted with

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).

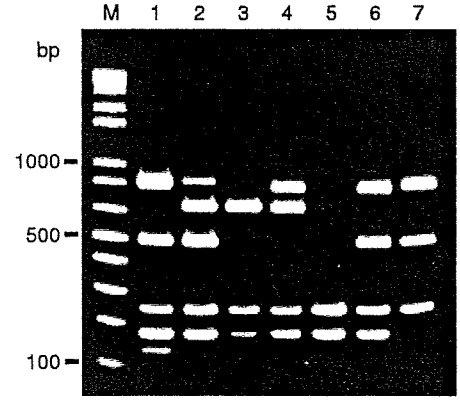

Figure 1 Agarose gel electrophoresis results for the multipler PCR assay with representative isolates of *Salmonella* subspecies. Lane 1, *S. enterica* subsp. I (NMJS1); Lane 2, *S. enterica* subsp. II (R42D2); lane 3, *S. enterica* subsp. IIIa (8M1); lane 4, *S. enterica* subsp. IIIb (WC1D1); lane 5, *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

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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NOTE *Pathology*

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

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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 nonpathogenic [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

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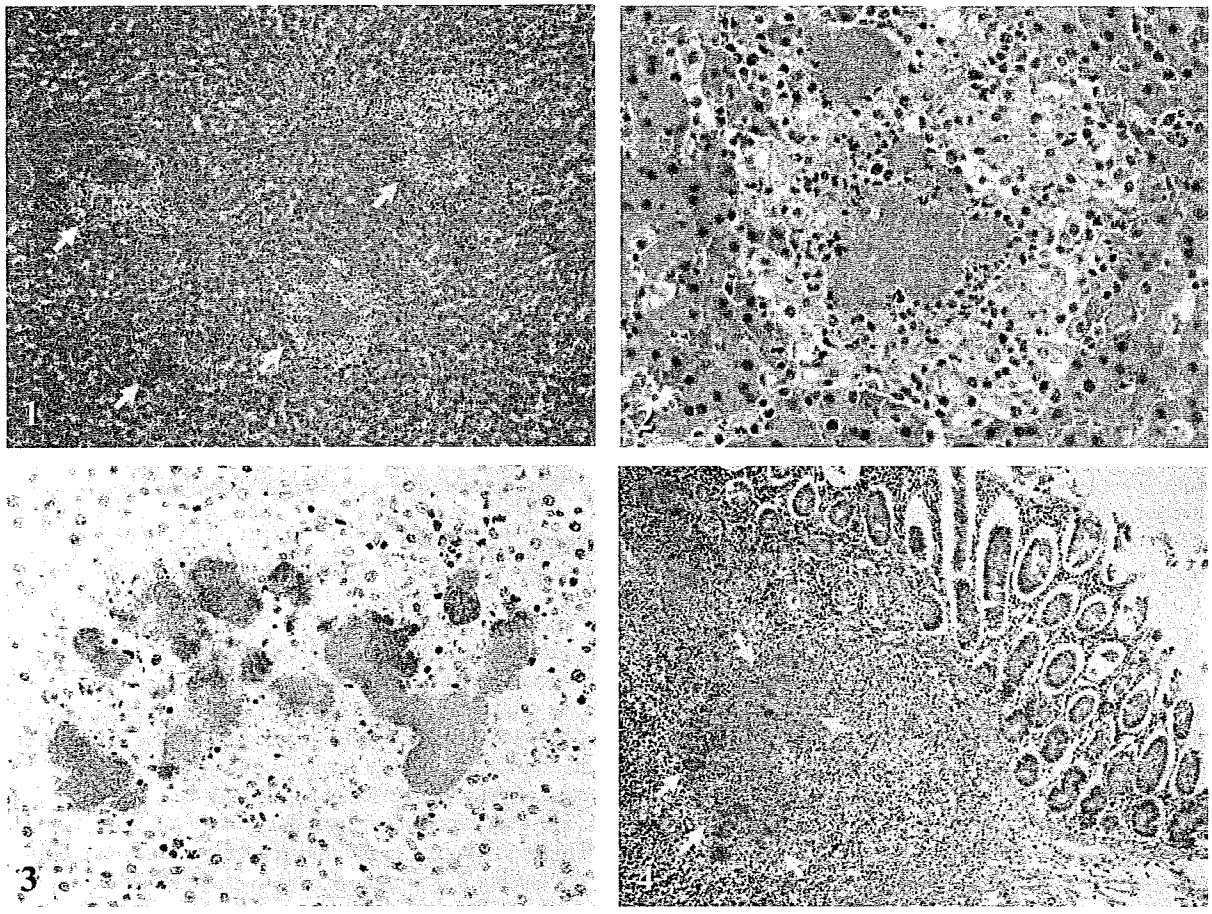

Fig. 1. Liver. Multi-focal necrosis accompanied by infiltration of neutrophils and macrophages included numerous bacterial colonies (arrows). Hematoxylin and Eosin (HE). × 100

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

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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

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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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 auto-cycling 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′-CCGTTACCGTTTTTACAATCGTTAATCAATGCTGATATGGAAAAGAGA-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.

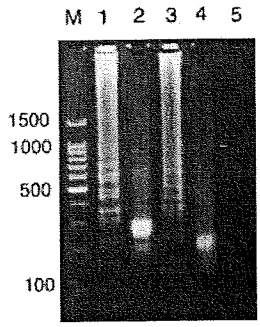

Fig. 1. DNA products from MPXV and camelpox virus amplified with COM-LAMP and separated in a 3% agarose gel by electrophoresis (**lanes 1** and **3**, respectively). To discriminate MPXV from camelpox, MPXV-LAMP and camelpox virus-LAMP products were treated with a restriction enzyme (*TaqI*) (**lanes 2** and **4**, respectively). A 100 bp-DNA ladder marker and negative control are also shown (**lanes M** and **5**, respectively).

infection was lethal in one naive monkey (Z-01-SC). The viremia level determined by the COM-LAMP assay continued to increase until sacrifice. The symptoms in the monkey pre-immunized 3 days before challenge (Z-03-SC) were less severe than those of the post-exposure vaccinated monkey (Z-02-SC). The viremia level in monkey (Z-02-SC) was significantly higher than that in monkey (Z-03-SC) throughout the observation period. The monkey pre-immunized 7 days before challenge (Z-04-SC) showed an asymptomatic infection. No viremia was demonstrated in this subject by the COM-LAMP assay (Fig. 2A). The severer the level of monkeypox-associated symptoms observed, the higher the viremia level determined by COM-LAMP. A similar phenomenon was observed in experiments in which monkeys were infected with MPXV Liberia through intranasal inoculation or the subcutaneous route at a dose of 10^6 PFU (Fig. 2B). The monkeypox symptoms in one monkey (L-03-SC) were so severe that the subject was sacrificed due to ethical considerations. The two monkeys (L-01-SC and L-020SC) infected with MPXV Liberia through the subcutaneous route showed moderately severe symptoms and survived. The two monkeys (L-04-IN and L-05-IN) showed mild symptoms with less than 20 papulovesicular skin lesions. Furthermore, viremia was demonstrated on Day 3 in the subcutaneously infected monkeys but not in the intranasally infected subjects.

Relationship Between Virus Loads as Determined by COM-LAMP and qPCR

The relationship between the virus load in the clinical samples determined by COM-LAMP and those determined by qPCR is shown in Figure 3. The correlation coefficient was 0.60, which represents a strong positive correlation.

DISCUSSION

Nucleic acid amplification-based diagnostic assays have become a gold standard for the rapid diagnosis of viral infections. Several PCR assays, such as conventional PCR and real-time quantitative PCR, have been reported for MPXV [Ibrahim et al., 1997; Neubauer et al., 1998; Kulesh et al., 2004; Aitichou et al., 2005, 2008; Saijo et al., 2006, 2008; Scaramozzino et al., 2007]. The real-time quantitative PCR assays have the advantages of rapidity, quantification-capacity, detection in a real-time manner, and high sensitivity. However, these nucleic acid amplification methods require high-precision instruments such as LightCycler instruments (Roche Diagnostics Ltd.). On the other hand, LAMP can be carried out without using such instruments. Furthermore, virus genomes can be detected within a shorter time and in a real-time manner. If turbidity detection is performed using a Loopamp real-time turbidmeter (LA-200), the virus genomes can be detected in a real-time manner along with genome quantification.

Three LAMP assays, COM-LAMP, C-LAMP, W-LAMP, were developed in this study. Using these assays, it was possible to detect the genomes of the Congo Basin and West African MPXVs and to differentiate between the genomes of the Congo Basin and of West African MPXVs by a combination of the three LAMP assays.

10^6 copies/reaction of the camelpox virus genome showed a positive reaction in the COM-LAMP (Table III), indicating that a positive reaction in the COM-LAMP assay does not always indicate an MPXV infection. The homology of the Loop-B-COM with

TABLE IV. Relationship Between the Results Obtained From the Nested PCR and LAMP Assays

	Samples from Zr-599-challenged monkeys Nested PCR		Samples from Liberia-challenged monkeys Nested PCR	
LAMP method	Positive	Negative	Positive	Negative
COM-LAMP				
Positive	21	0	24	0
Negative	3	24	8	40
C-LAMP				
Positive	19	0	0	0
Negative	5	24	33	39
W-LAMP				
Positive	0	0	23	0
Negative	24	24	9	40

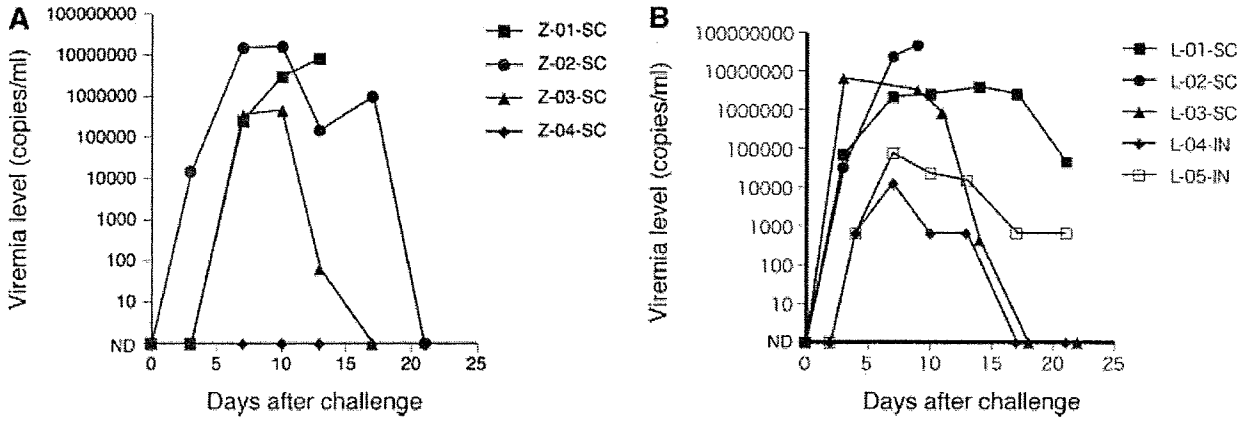

Fig. 2. Viremia level in peripheral blood collected from MPXV Zr-599-infected monkeys (**A**) and those in MPXV Liberia-infected monkeys (**B**) as determined by COM-LAMP. "ND" indicates it was below the detection level.

camelpox virus ATI-gene (80%) was higher than those with vaccinia virus and cowpox virus (76%), while there were no significant differences in homology of the other primers among these orthopoxviruses. Although further study is needed, the difference might be responsible for positive reaction of camelpox virus in COM-LAMP assay. Although a positive reaction in COM-LAMP does not indicate MPXV genome-positive, the amplified products of MPXV DNA and the other orthopoxvirus DNAs could be differentiated by restriction enzyme treatment or a combination of the COM-LAMP, C-LAMP and W-LAMP assays. Furthermore, when the viremia level determined by COM-LAMP was much less than that determined by C-LAMP, the samples can be understood to contain orthopoxviruses other than MPXV as orthopoxviruses such as camelpox and vaccinia viruses possesses a similar nucleotide sequence to the *D14L* gene of Congo Basin MPXV (data not shown). Although the data is not shown here, the genomes of herpes simplex virus and varicella zoster virus, which cause vesicular skin infections in humans and must be differentiated from human monkeypox, showed negative reactions in the newly developed LAMP assay. The corresponding genomes in variola virus, a causative agent for smallpox, to the partial ATI gene amplified by the COM-LAMP assay do not possess the *Taq*I restriction site, suggesting that the differentiation of MPXV from variola virus is possible by the COM-LAMP assay.

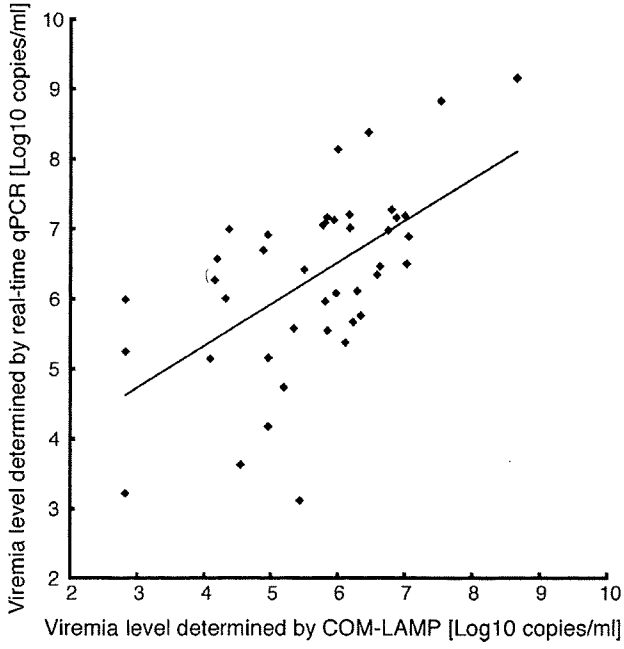

Fig. 3. Correlation between viral loads as determined by LAMP assays and real-time qPCR.

Three LAMP assays were evaluated in comparison with nested PCR. The sensitivity and specificity of the three LAMP assays when compared with the nested PCR were approximately 70–80% and 100%, respectively (Table IV). Because the nested PCR for the amplification of genome sequences is quite sensitive, the sensitivity of the LAMP assay was calculated to be 70–80% in this study. However, the sensitivity of the COM-LAMP assay was much higher when compared with the conventional PCR reported previously [Neubauer et al., 1998]. The viremia level determined by COM-LAMP was associated with the severity of clinical symptoms of monkeypox. The most significant advantages of LAMP assay over conventional PCR and real-time quantitative PCR is that the assay is simpler to perform. No highly specialized instruments are necessary for the LAMP assay, and even the detection of the turbidity derived from the accumulation of byproduct can be done visually [Mori et al., 2001]. It is concluded that the newly developed LAMP assays afford a valuable tool not only for the diagnosis of but also for the assessment of MPXV infections.

In summary, a sensitive, specific and rapid LAMP system for the detection of the MPXV genome was developed. Using this technology, MPXV can be differentiated into Congo Basin strains or West African

strains. This technology offers great benefits in the control of outbreaks of MPXV infections and in the assessment of the course of MPXV infections. Furthermore, the newly developed LAMP system may offer advantages in the diagnosis of human monkeypox, which would need to be differentiated from smallpox in the event of a variola virus-associated bioterrorism attack.

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The challenge experiments with MPXV were conducted in the high-containment laboratory at the NIID, Japan. All animal procedures were approved by the Committees on Biosafety and Animal Handling and Ethical Regulations of the National Institute of Infectious Diseases, Japan. Animal research was undertaken in compliance with the guidelines issued from the Ministry of Health, Labor and Welfare, "The Fundamental guidelines for proper conduct of animal experiment and related activities in institutions under jurisdiction (June 2006)." Our animal work also adhered to the principles stated in the guidelines.

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Characterization of Monoclonal Antibodies to Junin Virus Nucleocapsid Protein and Application to the Diagnosis of Hemorrhagic Fever Caused by South American Arenaviruses▽

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Junin virus (JUNV), Machupo virus, Guanarito virus, Sabia virus, and Chapare virus are members of New World arenavirus clade B and are the etiological agents of viral hemorrhagic fevers that occur in South America. In this study, we produced three monoclonal antibodies (MAbs) to the recombinant nucleocapsid protein of JUNV, designated C6-9, C11-12, and E4-2. The specificity of these MAbs was examined by enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence assay, and an epitope-mapping method. Using these MAbs, we developed antigen (Ag) capture ELISA systems. We showed that by using MAb C6-9, JUNV Ag was specifically detected. On the other hand, by using MAb C11-12 or E-4-2, the Ags of all human pathogenic South American arenaviruses were detected. The combined use of these Ag capture ELISA systems in the present study may be useful for the diagnosis of acute-phase viral hemorrhagic fever due to infection by a South American arenavirus.

The South American arenaviruses Junin virus (JUNV), Machupo virus (MACV), Guanarito virus (GTOV), Sabia virus (SABV), and Chapare virus (CHPV) are members of New World arenavirus clade B. JUNV, MACV, GTOV, and SABV are the etiological agents of Argentine hemorrhagic fever (AHF), Bolivian hemorrhagic fever (BHF), Venezuelan hemorrhagic fever (VHF), and Brazilian hemorrhagic fever, respectively (4). CHPV was also recently shown to be associated with cases of hemorrhagic fever in Bolivia (5). AHF emerged in the 1950s, and since then, outbreaks have occurred annually without interruption (4). The mortality rate for AHF is estimated to be 15 to 30%, but early treatment with immune plasma reduces the rate to less than 1% (6). The region at risk has been progressively expanding into northern central Argentina, and almost 5 million people are currently considered to be at risk for AHF (6, 13). Phylogenetic analysis indicates that JUNV is more closely related to MACV than to SABV or CHPV, whereas SABV and CHPV are more closely related to each other than to other New World arenaviruses (5).

Arenaviruses are enveloped and contain a bisegmented RNA genome. The genome consists of two ambisense single-stranded RNA molecules, one designated L, which encodes the RNA-dependent RNA polymerase and a zinc-binding matrix protein, Z, and the other designated S, which encodes the major structural components of the virion, i.e., the nucleocapsid protein (NP) and the envelope glycoprotein precursor (15). The arenavirus NP is the most abundant protein among the viral structural proteins both in infected cells and in virions (2) and is commonly used as a target for detecting viral antigens (Ags) (20). Moreover, arenavirus NPs have been known to be the most conserved among the same virus species and, to some extent, among different arenavirus species (3, 8). Therefore, it seems likely that monoclonal antibodies (MAbs) raised against the NP of an arenavirus would also be useful for detecting other arenaviruses (20). Recently, an immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) was developed by using a recombinant NP (rNP) of JUNV, obtained from a recombinant baculovirus system, and was proposed to be useful for etiologic confirmation of AHF in seroepidemiological studies (20, 26). It is considered that an Ag capture ELISA using MAbs specific for viral Ags allows rapid diagnosis of the acute phase of viral hemorrhagic fever by detecting viral Ags in blood or tissue homogenates (20). In this study, we produced MAbs to the rNP of JUNV. These MAbs were characterized by ELISA, indirect immunofluorescence assay (IFA), and an epitope-mapping method. Ag capture ELISAs were developed by using these MAbs that are specific for JUNV and that are broadly applicable for the detection of human pathogenic New World arenaviruses.

MATERIALS AND METHODS

Cell culture. Hybridomas and their parental cell line, P3/Ag568, were maintained in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids (Invitrogen), and antibiotics (streptomycin and penicillin G; Invitrogen). Hypoxanthine-aminopterin-thymidine supplement (Invitrogen) was added to the me-

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