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Short communication

Virulence characteristics of *Yersinia pseudotuberculosis* isolated from breeding monkeys in Japan

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Abstract

Between April 2001 and 2007, 18 *Yersinia pseudotuberculosis* outbreaks occurred in breeding monkeys at 12 zoological gardens in Japan, and 28 monkeys of 8 species died. A total of 18 *Y. pseudotuberculosis* strains from the dead monkeys, comprising one strain per outbreak, were examined for serotype and the presence of the virulence genes *virF*, *inv*, *ypm* (*ypmA*, *ypmB* and *ypmC*) and *irp2*. Of the 18 *Y. pseudotuberculosis* strains, 7 (38.9%) were serotype 4b, 7 (38.9%) were serotype 1b, and there was one each of serotypes 2b, 3, 6 and 7. All the 18 strains examined harbored *virF* and *inv*. Sixteen (88.9%) strains, including the strain of serotype 7, harbored *ypmA*. However, no strain harbored *ypmB*, *ypmC* and *irp2*.

This study demonstrated that among other pathogenic factors, almost all the *Y. pseudotuberculosis* isolated from the outbreaks had the *ypm* gene encoding the superantigenic toxin, YPM. As most of the monkeys who died in those outbreaks originated from South America and other regions, where the presence of the *ypm* gene have not been reported, YPM might be the cause, or at least the most important factor for, the high mortality of the breeding monkeys infected by *Y. pseudotuberculosis* in Japan. This is also the first report of a fatal case due to *Y. pseudotuberculosis* serotype 7 infection in the world.

Keywords: *Yersinia pseudotuberculosis*; Breeding monkey; Virulence genes; YPM

1. Introduction

Yersinia pseudotuberculosis is known to be an important causal agent of zoonosis. Monkey species are especially sensitive to *Y. pseudotuberculosis*, and many fatal cases of *Y. pseudotuberculosis* infection in

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breeding monkeys have been reported throughout the world, including in Japan (Buhles et al., 1981; Hirai et al., 1974; Kageyama et al., 2002; MacArthur and Wood, 1983; Maruyama et al., 1983; Murata and Hama, 1992; Rosenberg et al., 1980; Sasaki et al., 1996; Taffs and Dunn, 1983; Une et al., 2003). Affected monkeys may die unexpectedly or after a very short illness, and at the present time there is no effective preventive method against *Y. pseudotuberculosis* infection. Therefore, monkey *Y. pseudotuberculosis* infection poses a serious problem for zoological gardens engaged in monkey breeding.

The pathogenicity of *Y. pseudotuberculosis* is associated with several virulence factors. Pathogenic strains of *Y. pseudotuberculosis* harbor 70-kb virulence plasmid (pYV), which encodes a number of important virulence and virulence-associated proteins. Additionally, a high-pathogenicity island (HPI), encoding an iron uptake system represented by its siderophore yersiniabactin (Carniel, 1999), and *Y. pseudotuberculosis*-derived mitogen (YPM), which is a superantigenic toxin, are known to play important roles in causing severe systemic infection (Abe et al., 1997). However, it remains unclear which virulence factor is connected with the high mortality of monkeys in *Y. pseudotuberculosis* infection. In the present study, we investigated the characteristics of *Y. pseudotuberculosis* isolated from dead breeding monkeys in Japan.

2. Materials and methods

2.1. Bacterial strains

Eighteen *Y. pseudotuberculosis* strains isolated from monkeys that died in 18 outbreaks (one strain per outbreak) were analyzed. These outbreaks occurred between April 2001 and 2007 at 12 zoological gardens (A–L) in Japan, and a total of 28 monkeys of 8 species, comprising 19 squirrel monkeys (*Saimiri sciureus*), 2 hamadryas baboons (*Papio hamadryas*), 2 white-faced sakis (*Pithecia pithecia*), 1 agile gibbon (*Hylobates agilis*), 1 dusky leaf monkey (*Presbytis obscurus*), 1 orangutan (*Pongo pygmaeus*), 1 ring-tailed lemur (*Lemur catta*) and 1 ruffed lemur (*Varecia variegata*), died (Table 1). Pathological findings such as swelling of the Peyer's patch and abscesses in the spleen and liver were typical of yersiniosis. Outbreaks occurred two, three and four times in the zoological gardens C, H and G, respectively.

Table 1
Sources of *Y. pseudotuberculosis* isolated from breeding monkeys in Japan

No.	Strain	Institution	Region	Isolation month year	Source (number and species of other monkeys dead in the same outbreak)
1	NP011001	A	Kanto	April 2002	Squirrel monkey
2	NP031103	B	Kanto	November 2003	Orangutan
3	NP031101	C	Kanto	November 2003	Squirrel monkey (1 squirrel monkey)
4	NP050101	C	Kanto	January 2005	Squirrel monkey
5	NP070401	D	Kanto	April 2007	Dusky leaf monkey
6	NP031201	E	Kinki	December 2003	Squirrel monkey (2 squirrel monkeys)
7	NP040301	F	Chugoku	March 2004	Squirrel monkey
8	NP010401	G	Sikoku	April 2001	Squirrel monkey
9	NP030401	G	Sikoku	April 2003	Squirrel monkey
10	NP050102	G	Sikoku	January 2005	Squirrel monkey
11	NP051201	G	Sikoku	December 2005	Squirrel monkey
12	NP020501	H	Kyusyu	May 2002	Squirrel monkey
13	NP030601	H	Kyusyu	June 2003	Squirrel monkey
14	NP070201	H	Kyusyu	February 2007	Squirrel monkey
15	NP030701	I	Kyusyu	July 2003	Squirrel monkey (1 squirrel monkey)
16	NP050201	J	Kyusyu	February 2005	Hamadryas baboon (1 hamadryas baboon and 1 agile gibbon)
17	NP050301	K	Kyusyu	March 2005	Squirrel monkey (1 squirrel monkey)
18	NP050303	L	Kyusyu	March 2005	White-faced saki (1 white-faced saki, 1 ruffed lemur and 1 ring-tailed lemur)

2.2. Isolation and identification of Y. pseudotuberculosis

The samples (liver and spleen) collected from the dead monkeys were homogenized or suspended in phosphate-buffered saline (PBS: 7.2), and 10-fold serial dilutions of the suspension were plated on irgasan-novobiocin (IN) agar plates (Fukushima et al., 1990). These PBS suspensions were incubated at 4 °C for 3 weeks and then subcultured on IN agar plate after alkali (KOH) treatment (Aulisio et al., 1980). The plates were incubated at 25 °C for 48 h. Colonies morphologically similar to those of *Yersinia* spp. were subcultured on trypticase soy agar (TSA) (BBL, Sparks, MD, USA) and submitted for biochemical examination for identification, as described elsewhere (Wauters et al., 1988).

2.3. Serotyping

Serotyping of *Y. pseudotuberculosis* isolated from the monkeys was performed by slide agglutination with a commercial rabbit anti-*Y. pseudotuberculosis* sera set (Denka-Seiken Co., Tokyo, Japan), and with the rabbit immune sera made in our laboratory. Additional serotyping was performed by PCR as described by Bogdanovich et al. (2003).

2.4. PCR detection of virulence genes

Six sets of primers, designed in Table 2, were used for detection of the virulence genes *virF*, *inv*, *ypm* (*ypmA*, *ypmB* and *ypmC*) and *irp2*. The *virF* and *irp2* genes were used as the markers for the presence of pYV and HPI, respectively. Chromosomal DNA for PCR was isolated with a Wizard Genomic DNA Purification Kit (Promega Co., Madison, WI, USA) following the manufacturer's instructions. PCRs were performed in 50 μl volumes containing 5 μl of template DNA, 0.1 mM each of the four deoxynucleoside triphosphates, 5 μl of 10× PCR buffer, 3 mM $MgCl_2$, 0.1 μM of each primer, and 0.5 U of Taq DNA polymerase (Promega Co., Madison, WI, USA). The PCR amplifications were carried out at 94 °C for 5 min as an initial denaturation step and then subjected to 30 cycles consisting of 1 min at 94 °C, 1 min at 55 °C for detection of *virF*, *inv*, *ypmA* and *irp2*, or at 52 °C for detection of *ypmB*, or at 49 °C for *ypmC* (Table 2), 1 min at 72 °C, followed by a final 5 min extension step at 72 °C. Amplifications were performed with a Program Temperature Control System PC-701 (ASTEC, Fukuoka, Japan). Ten microliters of the PCR amplification products were subjected to electrophoresis in a 1.5% agarose gel. A 1-kb PLUS DNA Ladder (Invitrogen Co., Carlsbad, CA, USA) was used as a DNA size marker. The gels were stained with ethidium bromide for 10 min, and photographed under UV light.

3. Results

3.1. Serotyping of Y. pseudotuberculosis strains

By slide agglutination, 7 (38.9%) strains of the 18 were serotype 4b, 7 (38.9%) were serotype 1b, and

Table 2
Primers for PCR detection of virulence genes

Virulence factor	Taget gene	Sequence (5′–3′)	Annealing temperature (°C)	Size of product (bp)	Reference
pYV	*virF*	TCATGGCAGAACAGCAGTCAG ACTCATCTTACCATTAAGAAG	55	590	Wren and Tabaqchali (1990)
Inv	*inv*	TAAGGGTACTATCGCGGCGGA CGTGAAATTAACCGTCACACT	55	295	Nakajima et al. (1992)
YPMa	*ypmA*	CACTTTTCTCTGGAGTAGCG GATGTTTCAGAGCTATTGTT	55	350	Ito et al. (1995)
YPMb	*ypmB*	TTTCTGTCATTACTGACATTA TTTCTGTCATTACTGACATTA	52	453	Ramamurthy et al. (1997)
YPMc	*ypmA* and *ypmC*	ACACTTTTCTCTGGAGTAGCG ACAGGACATTTCGTCA	49	418	Carnoy and Simonet (1999)
HPI	*irp2*	AAGGATTCGCTGTTACCGGAC TCGTCGGGCAGCGTTTCTTCT	55	280	Schubert et al. (1998)

there was one each of serotypes 2b, 3, 6 and 7. *Y. pseudotuberculosis* serotype 7 has not been isolated from clinical samples in humans or in animals, and thus PCR-based serotyping was used to eliminate any doubt about the serotype of strain NP030601, which was identified as serotype 7 by the slide agglutination. The PCR result of strain NP030601 matched with the above condition for serotype 7 (data not shown), eliminating any doubt about the serotype of this strain. The results of the PCR-based serotyping of the other 17 strains also matched with those of the slide agglutination (data not shown). All the *Y. pseudotuberculosis* strains isolated from the monkeys who died in the same outbreak were of the same serotype of strains chosen for analysis in this study.

3.2. Detection of virulence genes in Y. pseudotuberculosis strains

All strains were *inv* and *virF* positive, and 16 (88.9%) of the 18 strains were *ypmA* positive by PCR. Of the 2 *ypmA* negative strains, one was serotype 4b, and another was serotype 3. On the other hand, all strains were *ypmB*, *ypmC* and *irp2* negative (Table 3).

Table 3
Characteristics of *Y. pseudotuberculosis* isolated from breeding monkeys

No.	Virulence genes						Serotype
	virF	*inv*	*ypm*			*irp2*	
			ypmA	*ypmB*	*ypmC*		
1	+	+	+	−	−	−	4b
2	+	+	+	−	−	−	4b
3	+	+	+	−	−	−	4b
4	+	+	+	−	−	−	4b
5	+	+	+	−	−	−	1b
6	+	+	+	−	−	−	4b
7	+	+	+	−	−	−	4b
8	+	+	+	−	−	−	1b
9	+	+	+	−	−	−	6
10	+	+	+	−	−	−	1b
11	+	+	+	−	−	−	2b
12	+	+	−	−	−	−	4b
13	+	+	+	−	−	−	7
14	+	+	+	−	−	−	1b
15	+	+	+	−	−	−	1b
16	+	+	−	−	−	−	3
17	+	+	+	−	−	−	1b
18	+	+	+	−	−	−	1b

+: PCR positive; −: PCR negative.

4. Discussion

In the present study, the predominant serotypes of *Y. pseudotuberculosis* isolated from dead monkeys were serotypes 1b and 4b. In Japan, these serotypes have also been the predominant serotypes isolated from clinical samples, for example, of human patients, and the majority of the strains of these serotypes are highly pathogenic, with the *ypmA* (Fukushima et al., 2001). In the present study, almost all of the strains isolated from dead monkeys also had *ypmA* genes. It is known that the presence of the *ypmA* is pretty much limited to the Far East (Japan, Korea and Far-Eastern Russia), and also that it exacerbates the toxicity of *Y. pseudotuberculosis* in systemic infection in mice (Fukushima et al., 2001). Moreover, it has been reported that the clinical signs of *Y. pseudotuberculosis* infection found in the Far East include not only fever, gastroenteric symptoms, and mesenteric lymphadenitis, which are the main symptoms in Europe, but also a variety of systemic manifestations such as rash, desquamation, erythema nodosum and arthritis (Sato et al., 1983). In zoological gardens in Japan, a variety of primates are bred, including monkey species from South America, Southeast Asia or Africa, listed in Table 1, as well as the Japanese macaque (*Macaca fuscata*). It has been noted that monkeys from those regions, where the presence of *Y. pseudotuberculosis* with the *ypm* gene has not been identified, frequently die when infections with this pathogen occur, while there has been little mortality of Japanese macaques due to *Y. pseudotuberculosis* infection (Kageyama et al., 2002). Because of the persistent exposure of the Japanese macaque to *Y. pseudotuberculosis* with the *ypm* gene from ancient times they may have acquired resistance to that pathogen, unlike the imported monkeys. Thus, YPM seems to be the main cause of the high mortality of the monkeys imported from abroad.

This is the first report of isolation of *Y. pseudotuberculosis* serotype 7 from a clinical sample anywhere in the world. This serotype has been isolated from dogs, raccoon dogs, moles, wild mice and water. However, there have been no reports about *Y. pseudotuberculosis* serotype 7 isolated from samples of primate origin. Pathological analysis of the squirrel monkey, from which the serotype 7 were isolated, showed swelling of the spleen and liver and multiple

white abscesses in the spleen, and the PCR analysis demonstrated that the strain of serotype 7 also harbored pYV and *ypmA* genes. These results possibly suggest that the strain serotype 7 isolated in the present study has the same degree of pathogenicity as the other pathogenic serotypes. Therefore, we should pay attention to the possibility of humans and other animal species infected by serotype 7.

Many monkey species kept at zoological gardens are formally recognized as ''threatened'' by The World Conservation Union (IUCN), and their deaths pose a serious loss to the zoological gardens involved. Thus, preventive measures against *Y. pseudotuberculosis* infection in breeding monkeys should be established as soon as possible. However, most breeding monkeys kept at zoological gardens are maintained in outdoor cages or enclosures for exhibition. These conditions lead to the exposure of the monkeys to animals living in the wild, such as birds and rodents, and as *Y. pseudotuberculosis* is widely distributed in wild animals, the probability of transmission of this pathogen from those animals is very high. Moreover, it is very difficult to completely prevent wild animals from invading the cages of the monkeys, and thus the foods and water provided for the monkeys can easily become contaminated. Therefore, development of effective vaccines is important for preventing pathogenic *Y. pseudotuberculosis* infection in breeding monkeys.

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