Seroepidemiological Survey of Pathogenic *Yersinia* in Breeding Squirrel Monkeys in Japan

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(Received 10 November 2009/Accepted 2 March 2010/Published online in J-STAGE 16 March 2010)

ABSTRACT. To investigate the prevalence of antibodies to pathogenic *Yersinia* in breeding squirrel monkeys, the serum samples of 252 squirrel monkeys from 9 zoological gardens in Japan were tested by ELISA using plasmid-encoded *Yersinia* outer membrane protein (Yops) as the antigen. The cutoff value was calculated by using the serum samples of the squirrel monkeys from Suriname, where no prevalence of pathogenic *Yersinia* have been reported. According to the cutoff value, 164 of 252 (65.1%) squirrel monkeys were considered positive against pathogenic *Yersinia*. These positive monkeys belonged to 8 of the 9 zoological gardens, and the percentage of the seropositive monkeys ranged from 22.2 to 89.4%. Furthermore, in one zoological garden, the positive rate of the squirrel monkeys which were over 1 year old (95.7%) was significantly higher than those which were under 1 year old (23.3%). These results suggested that pathogenic *Yersinia* is highly prevalent among breeding monkeys in Japan.

KEY WORDS: ELISA, squirrel monkey, *Yersinia*, Yops.

J. Vet. Med. Sci. 72(8): 981–984, 2010

Yersiniosis is an infection with pathogenic *Yersinia*, which is comprised of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis*. These pathogens cause gastrointestinal symptoms including enteritis, diarrhea and mesenteric lymphadenitis, and sometimes septicemia in humans and animals [3, 18]. Monkey species are especially sensitive to yersiniosis, and many fatal cases in breeding monkeys have been reported throughout the world [2, 8, 17, 23, 27]. In Japan, *Y. pseudotuberculosis* in particular frequently causes fatal infection in breeding monkeys [11, 14, 15, 30]. The highest number of dead monkeys by *Y. pseudotuberculosis* infection in Japan has occurred among the squirrel monkey (*Saimiri* spp.) [14, 30]. The habitat of the squirrel monkey is South and Central America, but many zoological gardens in Japan have been breeding monkeys imported from those regions. Many authors have published clinical and/or pathological reports of fatal infection with pathogenic *Yersinia* in breeding monkeys, including squirrel monkeys, but detailed information on the epidemiology of yersiniosis in breeding monkeys has not yet been obtained.

The pathogenicity of pathogenic strains of *Yersinia* depends on the presence of a 70-kb virulence plasmid termed “pYV”. This plasmid is essential for virulence and is used to differentiate pathogenic from nonpathogenic *Yersinia*. To establish infection and subvert host defenses, pathogenic *Yersinia* require a type III secretion system which translocates virulence factors, called Yops (*Yersinia* outer membrane proteins), into host cells [6, 25]. Some researchers have reported that enzyme-linked immunosor-

bent assay (ELISA) and immunoblot assays using Yops as antigen are a specific and sensitive method for detecting pathogenic *Yersinia* infection [22, 26, 28]. To determine the prevalence of pathogenic *Yersinia* infection in breeding monkeys, we conducted a seroepidemiological study in squirrel monkeys in Japan by ELISA using semi-purification Yops as antigen.

MATERIALS AND METHODS

Serum samples: Two hundreds fifty-two serum samples were collected from 9 zoological gardens (A–I) in Japan, and tested by ELISA for antibodies to Yops. In addition, 91 serum samples which were collected from Suriname immediately after importation were used as negative control. The serum samples were stored at –20°C until use, and inactivated at 56°C for 30 min before use.

Yops preparation: Yops were prepared according to the method of Heesemann *et al.* [12]. *Y. pseudotuberculosis* serovar 4b isolated from a dead squirrel monkey was precultured in BHI broth (Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.) at 25°C with shaking (110 rpm) overnight. This preculture was then diluted 1:20 with fresh BHI broth and incubated with shaking (110 rpm) at 37°C for 90 min. Filter-sterilized EGTA (Sigma, St. Louis, MO, U.S.A.) was added to the medium to final concentration of 2.5 mM, and incubation was continued for 90 min at 37°C. The bacterial cells were then removed by centrifugation (7,000 × g at 4°C for 20 min), and clarified culture supernatant was filter-sterilized. The proteins were precipitated from this culture supernatant by the addition of solid ammonium sulfate (40 g/100 ml of supernatant). The precipitated proteins were dissolved in distilled water, and dialyzed with

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Spectra/PoraCE Membrane MWCO:10,000 (Spectrum® Laboratories Inc., Rancho Dominguez, CA, U.S.A.). The retained volume was lyophilized and stored at -30°C until use.

SDS-PAGE: Yops were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The method used for SDS-PAGE was essentially the one described by Laemmli [16]. Briefly, Yops was suspended in Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.), boiled for 3 min, and then subjected to a 12.0% polyacrylamide gel. After that, the gel was stained with silver nitrate.

ELISA: ELISA was carried out in flat-bottom 96-well microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark). The plates were coated with $250\text{ }\mu\text{g}$ of Yops antigen/ml ($50\text{ }\mu\text{l}$ /well) in phosphate buffer saline (pH 7.2) and incubated overnight at 4°C . The wells were then blocked with Diluent/Blocking Concentrate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, U.S.A.) at 25°C for 15 min. In each of the three wells assigned for the individual serum sample, the wells were loaded with the sample (1:40 dilution in Wash solution; KPL) and incubated at 37°C for 1 hr. The plates were washed three times with Wash solution and incubated with peroxidase-conjugated Protein G (1:1,000 dilution in Diluent/Blocking Concentrate; Invitrogen Co., Carlsbad, CA, U.S.A.) at 25°C for 1 hr. After being washed five times, the plates were incubated with substrate ABTS (KPL) for 20 min at 25°C , and the optical density (OD) was measured at 405 nm by a MTP-120 microplate reader (Corona Electric Co. Ltd., Ibaraki, Japan).

Cutoff value: The OD values of 91 monkeys from Suriname, where no presence of pathogenic *Yersinia* have been reported [9], were considered to be a negative control. The cutoff value was calculated as the mean OD of the negative sera plus 3 standard deviations (SD). The Yops antibodies were considered positive when the OD value was higher than the cutoff value. The OD values of the 91 monkeys from Suriname ranged between 0.023 and 0.112, and the mean was 0.050 (Fig. 2). The SD was calculated to be 0.021 from those results. Therefore, the cutoff value was calculated to be 0.113.

RESULTS

SDS-PAGE analysis of Yops: The silver stained Yops showed 5 bands, and low background (Fig. 1). Designated bands (A-E) were considered to be YopH (51.0 kDa), YopB (41.8 kDa), YopD (33.3 kDa), YopN (32.6 kDa) and YopE (22.9 kDa), respectively [6, 21].

Prevalence of IgG antibodies to Yops in squirrel monkeys in Japan: Among the 252 squirrel monkeys tested, 164 (65.1%) showed an OD higher than the cutoff value, 0.113, and were therefore considered positive (Fig. 2). These positive monkeys belonged to 8 of the 9 zoological gardens, and the percentage of the seropositive monkeys ranged from 22.2 to 89.4% (Table 1).

Prevalence of serum antibody to Yops by age in squirrel

Fig. 1. SDS-PAGE followed by silver staining of the Yops. Left lane shows the prestained broad range protein molecular mass markers (Bio-Rad), and right lane shows Yops used as the antigen for ELISA.

Fig. 2. Antibody titers to Yops of 91 squirrel monkeys just after imported from Suriname. The vertical dashed line represents the cutoff value, which was calculated as 3 standard deviations (SD) from the mean of this group.

monkeys of institution H: All squirrel monkeys in institution H were individually recognized by electronic microchips, so that information about them, including the age, was controlled. To investigate the relationship between the age and prevalence of pathogenic *Yersinia*, the prevalence of antibody to Yops in institution H was arranged by age (Table 2). The positive rate of the monkeys that were over 1 year old (95.7%) was significantly higher than that under 1 year old (23.3%) ($P < 0.05$).

DISCUSSION

The present study demonstrated that pathogenic *Yersinia* is highly prevalent among breeding monkeys in Japan. Yops used as an antigen of ELISA are encoded in pYV, which is harbored in pathogenic strains of *Yersinia*. Regardless of the species and serovars of *Yersinia*, it is known that pathogenic *Yersinia* infection elicits specific antibody response to Yops in humans and animals [5, 13, 19]. Therefore, the squirrel monkeys considered Yops positive in the

Fig. 3. Analysis of sera obtained from 252 breeding squirrel monkeys in Japan. The vertical dashed line represents the cutoff value, which was calculated as 0.113.

Table 1. Prevalence of serum antibody to Yops in squirrel monkeys from 9 institutions in Japan

Region	Institution	No. of positive samples/ No. of samples tested (%)
Kanto	A	9/23 (39.1)
	B	12/15 (80.0)
	C	0/10 (0.0)
	D	2/9 (22.2)
Kinki	E	6/8 (75.0)
	F	6/23 (26.1)
Shikoku	G	11/14 (78.6)
Kyusyu	H	76/103 (73.8)
	I	42/47 (89.4)
Total		164/252 (65.1)

Table 2. Prevalence of serum antibody to Yops by age in squirrel monkeys of institution H

Age (years)	No. of positive samples / No. of samples tested (%)	Average OD value
< 1	7/30 (23.3)	0.084
1 – 2	13/15 (86.7)	0.300
2 – 3	18/18 (100.0)	0.420
3 – 4	3/4 (75.0)	0.389
4 – 5	10/10 (100.0)	0.494
5 – 6	7/7 (100.0)	0.559
> 6	16/16 (100.0)	0.420
Unknown	2/3 (66.7)	0.261
Total	76/103 (73.8)	0.315

present study must have been infected by pathogenic *Yersinia* in the past. However, squirrel monkeys that do not have any immunity to yersiniosis, such as infant monkeys, seem to die at a high rate when infected with *Y. pseudotuberculosis* considering past studies [14, 30].

Pathogenic *Yersinia* can be divided into low pathogenic

strains, which induce a mild intestinal infection in humans, and highly pathogenic strains, which cause severe systemic infection in humans [4, 10]. Whether pathogenic *Yersinia* causes limited gastroenteritis or systemic infection in humans correlate with the presence of a high-pathogenicity island (HPI), encoding an iron uptake system represented by its siderophore yersiniabactin [4] or *Y. pseudotuberculosis*-derived mitogen (YPM), which is a superantigenic toxin [1]. It is known that the presence of YPM is limited to the Far East (Japan, Korea and Far-Eastern Russia) [10], and in Japan, *Y. pseudotuberculosis* harboring YPM were isolated from almost all the fatal cases of breeding monkeys [14]. On the other hand, *Y. enterocolitica*, in particular serotype O3, O5,27, and O9 which are frequent causative agents of yersiniosis and do not harbor HPI, usually cause mild intestinal infection in humans [4, 24]. Maruyama reported that 10 Crab-eating Macaques (*Macaca fascicularis*) infected with *Y. enterocolitica* serotype O3 in experimental infection did not show any noteworthy clinical symptoms, except 3 which showed water diarrhea [20]. Each zoological garden keeps many squirrel monkeys, so even if the squirrel monkeys infected with these low pathogenic strains show the symptoms of yersiniosis, for example mild diarrhea, it is possible that those are passed over, or are not diagnosed as yersiniosis. These results suggested that the squirrel monkeys showing antibodies to Yops have been inapparently or mildly infected with low pathogenic strains of *Yersinia*, not highly pathogenic strains of *Yersinia* like YPM producing *Y. pseudotuberculosis*.

The zoological gardens which we investigated kept a number of squirrel monkeys, but did not collect sufficient information on each individual for our research purposes. However, institute H, which is located in the Kyusyu region and keeps the highest number of squirrel monkeys in Japan, individually recognizes all monkeys by electronic microchips. The microchips were implanted into all squirrel monkeys born in the years from 1997 to 2003, so the prevalence of serum antibody to Yops was arranged by age in Institute H. Almost all of the squirrel monkeys which were over 1 year old were positive, while the positive rate of those under 1 year old was only 23.3% (Table 2). These results suggest that the majority of breeding squirrel monkeys in Japan were probably infected by pathogenic *Yersinia* within one year of birth. As described above, in the present study, many squirrel monkeys that have never shown clinical signs of yersiniosis had the antibody to Yops. It is likely that inapparent infections of low pathogenic *Yersinia* frequently occur in breeding squirrel monkeys in Japan.

The present study demonstrated that pathogenic *Yersinia* is highly prevalent among breeding monkeys in Japan. Pathogenic *Yersinia* is a causal agent of zoonotic disease, and we cannot deny the possibility of human infection from monkeys. Therefore, from the point of view of public health, it is important to develop preventive methods to prevent pathogenic *Yersinia* infection in monkeys. However, as described above, pathogenic *Yersinia* strains are widely distributed in wild animals and livestock, so it is possible

that pathogenic *Yersinia* is distributed around zoological gardens. Many zoological gardens maintain breeding monkeys not only in indoor cages, but also outdoor cages or enclosures to which wild animals have easy access, so it is difficult to prevent pathogenic *Yersinia* infection in breeding monkeys even with proper attention to facility maintenance and sanitation, as well as feed hygiene. Therefore, development of an effective vaccine is important for preventing pathogenic *Yersinia* infection in breeding monkeys.

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