Identification of Pathogenic Strains within Serogroups of *Yersinia pseudotuberculosis* and the Presence of Non-Pathogenic Strains Isolated from Animals and the Environment

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ABSTRACT. The existence of apathogenic strains of *Yersinia pseudotuberculosis* (Yp) has not so far been reported. Recently, the authors characterized new serogroups and a new subgroup in Yp, that is, O9, O10, O12, O13, O14 and O1c, and the pathogenicity of these new strains was of interest. A total of 137 strains of serogroups O1c, O6, O7, O9, O10, O11, O12, O13 and O14 of Yp were investigated for their pathogenicity *in vivo* and *in vitro*. Although catalase activity and the *inv* gene were detected in all strains except those of groups O13 and O14, only a few strains, from serogroups O6 and O10 caused severe infection in mice. The remaining strains caused no mortality or severe infection even when they grew in limited tissues of infected mice. All the strains of Yp not possessing the virulence plasmid pYV caused no severe infection in mice. It is evident that less pathogenic Yp exists and that not only pathogenic but also less pathogenic Yp organisms exist in the same serogroup. — **KEY WORDS:** new serogroup, non-pathogenic strain, *Yersinia pseudotuberculosis*.

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Yersinia pseudotuberculosis has been classified into serogroups O1 to O8 [12–14, 19, 23, 26]. Recently, new serogroups O9, O10, O11 and a new subgroup O1c were added by Tsubokura and Aleksic [24]. Bacteria belonging to these serogroups were not isolated from diseased animals and patients but from wild rats, racoon dogs and mountain water [24]. More recently, further serogroups, designated O12, O13 and O14, were added by the present authors [in press]. *Yersinia enterocolitica* has been classified into 27 O-serogroups and 5 biotypes [2, 29, 30]. It is well known that the serogroup and biotype of pathogenic *Y. enterocolitica* strains are limited and most non-pathogenic strains of *Y. enterocolitica* has been isolated from wild animals and the environment [30, 31]. Numerous strains of *Y. pseudotuberculosis* which belong to serogroups O1 to O5 have been isolated in Europe and Japan and almost all of them are pathogenic [12, 13, 19, 23, 25, 27]. There are several reports on the virulence factors of *Y. pseudotuberculosis*, however only limited information on non-pathogenic strains. Tsubokura *et al.* isolated an avirulent strain of *Y. pseudotuberculosis* O3 from swine which does not ferment melibiose [25]. The relationships between pathogenicity and serological and biochemical properties of *Y. pseudotuberculosis* have been poorly characterized. It is possible that avirulent *Y. pseudotuberculosis* strains as well as *Y. enterocolitica* are isolated from humans, animals and/or the environment. We were interested to survey avirulent strains of *Y. pseudotuberculosis*. In the present investigation, newly serogrouped strains of *Y. pseudotuberculosis* were examined for their pathogenicity *in vivo* and *in vitro* since some of

them were not isolated from patient or diseased animals, but from apparently healthy free-living animals and the environment.

MATERIALS AND METHODS

The strains of *Y. pseudotuberculosis* used in this investigation are listed in Table 1. The *in vitro* tests to select avirulent strains of *Y. pseudotuberculosis* were performed as follows. An auto-agglutination test at 37°C was done by the method of Laird and Cavanaugh [14] modified using MR-VP medium (Difco Laboratories, Detroit, Mich.). The calcium dependent test at 37°C was carried out on magnesium oxalate agar: trypticase soy agar plate supplemented with 20 mM sodium oxalate and 20 mM magnesium chloride [8]. Pyrazinamidase activity was tested by the method of Kandolo and Wauters [10]. Catalase activity was detected on trypticase soy slant agar (BBL, Microbiology Systems, Cockeysville, Md.) [1]. After incubation for 48 hr at 25°C or 37°C, one milliliter of a 3% hydrogen peroxide solution was flooded onto the slant. The tubes generating a considerable amount of oxygen were recorded as catalase-positive. Possession of plasmid was identified by the alkaline lysis technique of Kaneko *et al.* [11], followed by agarose gel electrophoresis for 0.5 hr at 100 V on a 0.7% horizontal agarose gel in Tris-borate buffer. Restriction endonuclease analysis of plasmid DNA was carried out with *Bam*HI [11]. Detection of *virF* gene encoded in plasmid DNA and of *inv* gene encoded by chromosomal DNA as a virulence-associated gene, was performed with the polymerase chain reaction (PCR) method described by Nakajima *et al.* [16]. The primers for the *virF* gene and *inv* gene were obtained from Dr. H. Nakajima (Okayama Prefecture Institute for Environmental Science and Public Health, Okayama, Japan).

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Table 1. The strain used

Serogroup	No. of strain	Source
1c	2	Wild-living small rodent, mountain water
6	45	Dog, racoon dog, guinea pig, wild-living small rodent, mountain water
7	46	Dog, racoon dog, wild-living small rodent, mountain water
9	14	Wild-living small rodent, mountain water
10	16	Racoon dog, mountain water, chicken
11	7	Mole, wild-living small rodent, mountain water
12	4	Mountain water
13	2	Wild-living small rodent
14	1	Wild-living small rodent
Total 137		

Pathogenicity for mice was also examined. Briefly, groups of five ICR mice (female, 4 weeks old) deprived of water for 18 hr were allowed to drink freely 25 ml of water containing 10^9 cfu/ml of one of the bacterial strains. Infected mice were observed for clinical signs and for recovery of bacteria from 1 to 12 days post inoculation (p.i.). The mice that died during the experimental period were autopsied immediately, survivors were killed and autopsied at 12 days p.i. The lung, liver, spleen, kidney, mesenteric lymph nodes and intestine were removed from each mouse, plated on CIN agar plate (Difco Laboratories, Detroit, Mich.) and incubated at 25°C. Colonies were

counted in 48 hr p.i.

RESULTS

Detection of virulence-associated properties among Y. pseudotuberculosis of different serogroups: The results obtained are summarized in Table 2. A few strains belonging to serogroups O1c, O6, O7, O10, O12 and O14 were positive in the auto-agglutination test; a few strains belonging to serogroups O6, O7 and O10 showed calcium dependency; all strains except O13 and O14 ones showed catalase activity. Some strains belonging to O6 and O10

Table 2. Patterns of virulence-associated properties with serogroups of *Y. pseudotuberculosis*

Serogroup	AA ^{a)}	Ca ²⁺ ^{b)}	PZ ^{c)}	Cat ^{d)}	pYV ^{e)}	inv ^{f)}	virF ^{g)}	Mouse ^{h)}	No. of strain
1c	+	-	+	+	-	+	-	-	1
	+	-	-	+	-	+	-	-	1
6	+	+	d ⁱ⁾	+	+	+	+	+	3
	+	D ^{j)}	d	+	-	+	-	-	5
	-	-	+	+	-	+	-	-	37
7	+	+	-	+	-	NT ^{k)}	NT	NT	1
	-	+	D	+	-	+	-	-	29
	-	-	D	+	-	+	-	-	16
9	-	-	+	+	-	+	-	-	14
10	+	D	-	+	+	+	+	+	4
	-	D	-	+	+	+	+	+	9
	-	+	+	-	+	-	NT	3	
11	-	-	D	+	-	+	-	-	7
12	d	-	d	+	-	+	-	-	4
13	-	-	-	-	-	+	-	-	2
14	+	-	-	-	-	+	-	-	1

a) AA; auto-agglutination. b) Ca²⁺; calcium dependence. c) PZ; pyrazinamidase activity. d) Cat; catalase activity. e) pYV; presence of virulence plasmid. f) *inv*; presence of *inv* gene. g) *virF*; presence of *virF* gene. h) Mouse; virulence to mice. i) d; negatives were majority. j) D; positives were majority. k) NT; not tested.

Fig. 1. Restriction endonuclease *Bam*HI assay of plasmids from *Y. pseudotuberculosis* O1c, O6, O7, O9, O10 and O11.

possessed the virulence-associated factors.

Plasmid DNA in different serogroups of *Y. pseudotuberculosis*: Most strains investigated possessed plasmids, however the virulence plasmid was detected only in a few O6 and O10 strains (data not shown). The restriction endonuclease assay of plasmid DNA with *Bam*HI (Fig. 1) gave patterns that divided the strains into 9 types, type A to type I. Type D was most prevalent and type G was detected in all serogroups tested except O10. Types A and B had identical patterns of virulence plasmid whereas type C to type I had non-pathogenic patterns (Fig. 1). The gene *virF* was detected in the strains which harbored pYV from groups O6 and O10. The *inv* gene was demonstrated in the strains of all serogroups in the PCR test (Table 2 and Fig. 2).

Pathogenicity of *Y. pseudotuberculosis* for mice: Pathogenicity of *Y. pseudotuberculosis* for mice was calculated by investigating mortality and multiplication of bacteria in the intestine and other organs. We considered a strain of *Y. pseudotuberculosis* as pathogenic for mice when more than 10^7 cfu/g of bacteria was continually recovered from feces throughout this investigation and the bacteria were recovered from other organs at 12 days p.i. A few

strains of serogroups O6 and O10 were lethal for mice. These strains grew in the intestine of infected mice and high numbers of bacteria were recovered from feces throughout the experiment. Moreover, the spleens were enlarged and many different-sized grayish-white foci were present in the spleen and the liver. Large numbers of bacteria were recovered from the lung, liver, spleen and kidney of infected mice. While, in mice inoculated with strains of serogroups O1c, O6, O7, O9, O11 and O12, the number of bacteria recovered from the feces gradually decreased, and in most mice no macroscopic lesions were seen in any organs, except from some of the mesenteric lymph nodes. No infecting bacteria were recovered from these organs. The duration of excretion of strains of O1c and O9 was less than that of other serogroups. Few bacteria belonging to serogroups O13 and O14 were recovered from feces of inoculated mice at 2 days p.i. (Table 3).

Summary of the pathogenicity of different serogroups of *Y. pseudotuberculosis*: The pathogenicity of different serogroups of *Y. pseudotuberculosis* was investigated (Table 4). The pathogenicity of strains of serogroups O1 to O5 was not examined in this study, as they have been isolated from patients and their pathogenicity has already been

Fig. 2. Detection of *virF* gene and *inv* gene from *Y. pseudotuberculosis* by polymerase chain reaction. A and B, O1c; C and D, O6; E and F, O7; G and H, O9; I and J, O10; K and L, O11; M and N, O12; O and P, O13; Q, O14.

Table 3. Comparison of mouse pathogenicity with serogroups of *Y. pseudotuberculosis*

Serogroup	Colonization of intestine	Propagation in organ	Lethality
1c	+	—	—
	—	—	—
6	+	+	+
	+	—	—
7	+	+	—
	+	—	—
9	—	—	—
10	+	+	+
	+	+	+
11	+	—	—
12	+	—	—
13	—	—	—
14	—	—	—

Table 4. Pathogenicity of *Y. pseudotuberculosis* with serogroups

Serogroup	Pathogenicity
1 1a	+
1b	+
1c	—
2 2a	+
2b	+
2c	+
3	+
4 4a	+
4b	+
5 5a	+
5b	+
6	+,—
7	—
9	—
10	+,—
11	—
12	—
13	—
14	—

confirmed [17]. Some strains tested in this investigation, belonging to serogroups O6 and O10, were pathogenic while no strains belonging to serogroups O1c, O7, O9, O11, O12, O13 and O14 were severely pathogenic.

DISCUSSION

In this investigation, we showed that avirulent strains of *Y. pseudotuberculosis* exist. New strains serogrouped as O1c, O7, O9, O11, O12, O13 and O14 may be able to be classified as avirulent organisms when more strains of other serogroups have been tested for their pathogenicity.

We distinguished between pathogenic and less-pathogenic *Y. pseudotuberculosis* by the possession of pYV plasmid [3–6, 9, 18] and the results of experimental infection of mice. Only a few strains belonging to O6 or O10 serogroups were positive in both tests and they can be considered as pathogenic. The pYV plasmid controls production of YOPs of *Yersinia* organisms. Loss of pYV plasmid or point mutation in the pYV plasmid results in decreased pathogenicity [3–6, 9, 18]. On the other hand, Miller *et al.* [15] reported that pathogenicity of *Yersinia* organisms is also encoded by chromosomal DNA. In fact, Shimonet *et*

al. [20, 21] observed that a strain of *Y. pseudotuberculosis* lacking pYV plasmid was able to multiply in tissues at the early stages of infection, similarly to strains harboring the pYV plasmid. Loss or mutation of the pYV plasmid induced a granulomatous response [22, 28]. Fukushima *et al.* [7] reported that *Y. pseudotuberculosis* O4a lacking pYV was pathogenic and naturally infected humans. Thus, in *Yersinia* organisms, pathogenicity is governed by virulence factors encoded by chromosomal DNA and virulence factors of the pYV plasmid are linked to chromosome. Although pathogenicity of strains of *Y. pseudotuberculosis* lacking a pYV plasmid are attenuated, these are still enteropathogenic for mice because of the virulence factor encoded by chromosomal DNA. It is evident that invasiveness for epithelial cell is controlled by a virulence factor encoded by chromosomal DNA of *Yersinia* organisms. Nevertheless, non-pathogenic strains identified in this study are not enteropathogenic for mice. These observations suggest that pathogenicity of *Y. pseudotuberculosis* is governed by virulence factors other than invasive ability and the other factors are encoded by the pYV plasmid. In this study, it was shown that catalase activity of O1c, O7, O9, O11 and O12 strains is closely related to their ability to colonize the intestine. It is possible that non catalase producing bacteria are unable to multiplying *in vivo*, therefore strains of O13 and O14 could not colonize the intestine and invade through mesenteric lymph nodes.

Pathogenicity of *Y. enterocolitica* is variable [2, 29–31] and pathogenic strains belong to limited serogroups and biogroups. Although non-pathogenic *Y. pseudotuberculosis* has not been recognized, non-pathogenic strains of *Y. pseudotuberculosis* are present in free-living animals and the environment. It is unclear why no non-pathogenic strain of *Y. pseudotuberculosis* has been recovered until now. Both non-pathogenicity and poor distribution make it difficult to isolate avirulent strains but a recent survey of *Yersinia* organisms proved their existence. The present results had new light on the study of ecology in *Yersinia* organisms.

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