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Restriction Endonuclease Analysis of Virulence Plasmids for Molecular Epidemiology of *Yersinia pseudotuberculosis* Infections

HIROSHI FUKUSHIMA,[1]* MANABU GOMYODA,[1] SEIJI KANEKO,[2] MISAO TSUBOKURA,[3] NOBUAKI TAKEDA,[4] TOSHIHARU HONGO,[4] AND FELIX NIKOLAEVICH SHUBIN[5]

Public Health Institute of Shimane Prefecture, Matsue, Shimane 690-01,[1] Tokyo Metropolitan Research Laboratory of Public Health, Shinjyuku-ku, Tokyo 160,[2] Department of Veterinary Microbiology, Faculty of Agriculture, Tottori University, Tottori, Tottori 680,[3] and Kurashiki Central Hospital, Kurashiki, Okayama, 710,[4] Japan, and Institute of Epidemiology and Microbiology, Academy of Medical Science Siberian Branch, 690600 Vladivostok, Russia[5]

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Restriction endonuclease analysis of virulence plasmid DNA was used to study the epidemiology of *Yersinia pseudotuberculosis* infections. The origin of *Y. pseudotuberculosis* could be divided into two focus areas: Eastern Asia and Europe. Wild animals were an important reservoir for the *Y. pseudotuberculosis* seen in infections in humans.

Yersinia pseudotuberculosis causes sporadic and epidemic infection in humans and has a wide distribution among domestic pets, farm animals, wild animals, soil, and fresh water (18). A detailed epidemiology of *Y. pseudotuberculosis* infections, especially modes of transmission to humans and genetic correlations of organisms in various areas of the world, has not been documented.

The virulence of *Y. pseudotuberculosis* is associated with the harboring of a 40- to 50-MDa virulence plasmid (9). Our groups (11) previously examined Japanese isolates to determine whether restriction endonuclease analysis of virulence plasmid DNA (REAP) might serve as an epidemiological marker. Almost all of the isolates harbored a single 40- to 50-MDa plasmid, and REAP elucidated possible routes of transmission of *Y. pseudotuberculosis* infection to humans. In the present study, a large number of isolates obtained from various countries were examined.

Six hundred eighty-seven *Y. pseudotuberculosis* strains belonging to serotypes 1a, 1b, 2b, 2c, 3, 4a, 4b, 5a, 5b, 6, 7, and 10 were from patients, wild and domesticated animals, food, and environmental sources in Japan, Russia (Far East region), Canada, Australia, New Zealand, Germany, Belgium, and Italy (Table 1). The plasmids were investigated by the alkaline lysis technique of Kaneko and Maruyama (10) followed by agarose gel electrophoresis for 0.5 h at 100 V on a 0.7% horizontal agarose gel in Tris-borate buffer. REAP was carried out with *Bam*HI, *Eco*RI, *Xho*I, *Pst*I, and *Hae*III. The resulting digests were analyzed by electrophoresis for 2 h at 100 V on a 0.7% horizontal agarose gel in Tris-borate buffer.

REAP of these strains revealed 16 (A to P) distinct restriction patterns with *Bam*HI (Table 2; Fig. 1). The serotypes which showed the same restriction patterns with *Bam*HI and *Eco*RI were then restricted with *Xho*I, *Pst*I, and *Hae*III. The composite REAP using five enzymes revealed 29 distinct patterns. REAP of serotypes showed one to seven distinct patterns. The composite REAP pattern of each serotype could be represented by the REAP pattern with *Bam*HI. A comparison of the geographic distribution of REAP patterns of each serotype is shown in Table 1. The serotype 1a strains showed two REAP patterns; REAP pattern A was observed in Russian isolates, and pattern B was seen in samples from Europe and Australasia. The serotype 1b, 2b, and 3 isolates from Europe and Australasia also showed identical REAP patterns for each serotype. However, these REAP patterns were rare in isolates from Eastern Asia and North America. The REAP pattern of serotype 1b isolates from Canada was same as that of Japanese isolates, and REAP patterns of serotype 1b, 3, 4a, and 4b isolates from Russia were also the same as those of Japanese isolates. However, REAP patterns of Canadian and Russian strains were not observed in the isolates from Europe and Australasia. This is the first documentation of different geographic distributions of *Y. pseudotuberculosis*. These results show that the origin of *Y. pseudotuberculosis* can be divided into two focus areas; eastern Asia and Europe. The origin of most *Y. pseudotuberculosis* in Japan and North America may be the Far East region of the Eurasia continent, because the islands of Japan and the North American continent were connected to the Eurasia continent before the Ice Age. This hypothesis was supported by the presence of identical REAP patterns among Russian, Japanese, and Canadian isolates belonging to serotypes 1b, 3, and 4a. This postulation is also supported by the differences in REAP patterns among serotype 1a, 1b, and 2b isolates in eastern Asia and Europe. REAP revealed that all strains belonging to serotypes 1a, 1b, and 2b isolated in Europe and Australasia had identical profiles for each serotype; these REAP patterns were rarely prevalent in eastern Asia and North America. These results confirmed that *Y. pseudotuberculosis* in Australasia may have been unknowingly introduced by human carriers or in shipments of domestic livestock from Europe and South Africa in the late 1700s and early 1800s, as discussed by Slee and Skilbeck (14).

A comparison of REAP patterns of isolates from patients, pork, animals, and environmental sources is shown in Table 1. Almost all REAP patterns of isolates from dogs, cats, pigs, pork, cattle, and humans were identical. Some investigators noted that human *Y. pseudotuberculosis* infections followed the infection of a family pet such as a dog or cat (5, 12). The REAP pattern of isolates in a familial outbreak showed evidence for the transmission of *Y. pseudotuberculosis* through contamination by feces from cats infected with these species (Table 3) (5). These findings confirmed that family pets such as cats and dogs are a major reservoir of *Y. pseudotuberculosis*.

* Corresponding author. Mailing address: Public Health Institute of Shimane Prefecture, 582-1 Nishihamasada, Matsue, Shimane 690-01, Japan. Phone: 0852-36-8181. Fax: 0852-36-6683.

TABLE 1. REAP patterns by *Bam*HI among serotypes isolated from various sources in Asia, North America, Europe, and Australasia[a]

Country	Source	No. of strains total	No. of strains (with indicated REAP pattern by *Bam*HI) of serotype:																																		
			1a		1b						2b					2c		3				4a		4b							5a			5b		6(B)	7(I)	10(D)
			A	B	B	D	E	F	H	O	B	D	H	I	J	G	I	B	C	D	N	B	L	B	D	G	H	K	L	M	B	H	I	I	P			
Japan	Dog	17				3		4												1				1	7											1		
	Cat	4				2												1						1														
	Soil	2				1												1																				
	Sand	2				1												1																				
	Cow	1											1																									
	Pig	87				5		11		2			5			8		21		3	7			17		1		7										
	Pig carcass	3														1				1								1										
	Pork	1																								1												
	Human	226				16	3	2	3	1			4	19			5	3		2	1			11	8	57	4	7			1	4	23	51	1			
	Well water	40				1								3			1									9		1					5	18			2	
	Mountain water	76				16			1				1					2					1	21		28			5							1		
	Raccoon dogs	100			2	4					1	1	8	3		1	2	3		7		2		17	10	8		5	3	1			5	12		2		3
	Wild mice	10				1																3				6												
	Deer	7																1								2			4									
	Other wild mammals[b]	12				1							2							2	5			1									1					
	Wild duck	2			1																					1												
Russia	Human	34				5												12				1			16													
	Spaghetti	1																							1													
	Kitchen[c]	2																							2													
	Vegetables[d]	6																1	1			1			3													
	Soil[e]	2																				2																
	Wild mice	13																	2			10			1													
	Wild rat	4																4																				
	Wild duck	1																				1																
	Reindeer	3	3																																			
	Salmonidae	2	2																																			
Canada	Human	2			2																																	
Belgium	Human	6		3			2														1																	
Germany	Human	1																			1																	
	Cat	1		1																																		
	Hare	4		1			1				1				1																							
	Duck	1					1																															
Italy	Pigeon	3		3																																		
	Guinea pig	1		1																																		
	Duck	1		1																																		
New Zealand	Deer (domestic)	2		1							1																											
Australia	Cow	3		1											2																							
	Deer (domestic)	4		1			1				1										1																	
Total		687	5	13	5	56	8	17	4	3	4	1	21	25	3	10	8	49	3	16	16	20	1	69	48	113	4	21	12	1	1	4	34	81	1	4	2	3

[a] The strains were kindly provided by S. Aleksick, A. Borczyk, E. de Boer, T. Hongou, T. Honda, M. Inoue, S. Kaneko, J. Katayama, R. Robins-Brown, M. Sasaki, F. N. Shubin, Y. Toyokawa, M. Tsubokura, and R. Van Noyen.

[b] Hare, wild boar, squirrel, and bonnet (one each); fox (two); and marten and monkey (three each).

[c] Table and utensils in the kitchen.

[d] Cabbage and beet (one each) and potatoes and onions (two each).

[e] Soil from potatoes.

TABLE 2. REAP patterns of 687 *Y. pseudotuberculosis* isolates

REAP pattern with:						No. of isolates												Reference strain(s) (serotype donor)[a]
All 5 enzymes (composit)	*Bam*HI	*Eco*RI	*Xho*I	*Pst*I	*Hae*III	of serotype:											Total	
						1a	1b	2b	2c	3	4a	4b	5a	5b	6	7	10	
1	A	1	ND[b]	ND	ND	5												5	488 (1a, S)
2	B	2	1	1	1	13												13	Verpoest (3, V)
3	B	2	2	1	2		5			50								55	RD43 (1b, F); Pa3423 (3, F)
4	B	2	2	2	3										4			4	DD110 (6, F)
5	B	2	2	2	2								1					1	84-406 (5a, K)
6	B	2	3	1	1			4										4	BB28 (2b, R)
7	B	3	3	3	3						20							20	149 (4a, T)
8	B	3	3	4	2							68						68	Pa 3422 (4b, F)
9	C	4	ND	ND	ND					3								3	354 (3, S)
10	D	5	4	5	2		56											56	Pa1994 (1b, F)
11	D	5	5	1	4			1										1	5597 (2b, I)
12	D	6	4	5	2					16								16	D585 (3, F)
13	D	7	4	6	4							48						48	Wla643 (4b, F)
14	D	7	4	6	2												3	3	6088 (10, I)
15	E	8	ND	ND	ND		8											8	Pa3492 (1b, F)
16	F	9	ND	ND	ND		17											17	Pa9198 (1b, F)
17	G	10	5	7	3				10			113						123	PT504 (2c, F); Pa3450 (4b, F)
18	H	11	ND	ND	ND		4											4	Pa12116 (1b, F)
19	H	12	ND	ND	ND			21										21	PT94 (2b, F)
20	H	13	ND	ND	ND							4						4	87-11 (4b, K)
21	H	14	ND	ND	ND								4					4	A146 (5a, K)
22	I	15	6	8	5			25	8				34	81		2		150	511 (2b, T); 5845 (2c, I); 5592 (5a, I); 492 (5b, T); K186 (7, H)
23	J	16	ND	ND	ND			3										3	G5431 (2b, R)
24	K	17	ND	ND	ND							19						19	143 (4b, K)
25	L	18	7	9	3						1	15						16	MW993-1 (4a, F); W134 (4b, F)
26	M	19	ND	ND	ND							1						1	81-286 (4b, K)
27	N	20	ND	ND	ND					16								16	Vlassel (3, V)
28	O	21	ND	ND	ND		3											3	510-1 (1b, K)
29	P	22	ND	ND	ND									1				1	K38 (5b, H)
Total						18	93	54	18	85	21	268	39	82	4	2	3	687	

[a] Donors: H. Fukushima (F), S. Kaneko (K), T. Hongou (H), R. Van Noyen (V), R. Robins-Brown (R), M. Tsubokura (T), M. Inoue (I), and F. Shubin (S).
[b] ND, not determined.

The presence of *Y. pseudotuberculosis* in farm animals such as pigs and cattle has been reported elsewhere (8, 17). Studies in Europe, Canada, and Japan have shown that pigs are an important reservoir of *Y. pseudotuberculosis* (15, 18), and an association between clinical human infections and pork ingestion has been discussed previously (16). Our survey of slaughtered pigs (7) and retail meat (2) revealed that 0.25% of pig carcasses and 0.8% of ground pork were contaminated with *Y. pseudotuberculosis*. In this study, identical REAP patterns within each serotype from pigs and pig carcasses in the same slaughterhouse support our hypothesis that the contamination of retail pork with *Y. pseudotuberculosis* followed the contamination of pig carcasses at slaughter (7). However, the link between a pig reservoir and transmission to humans remains

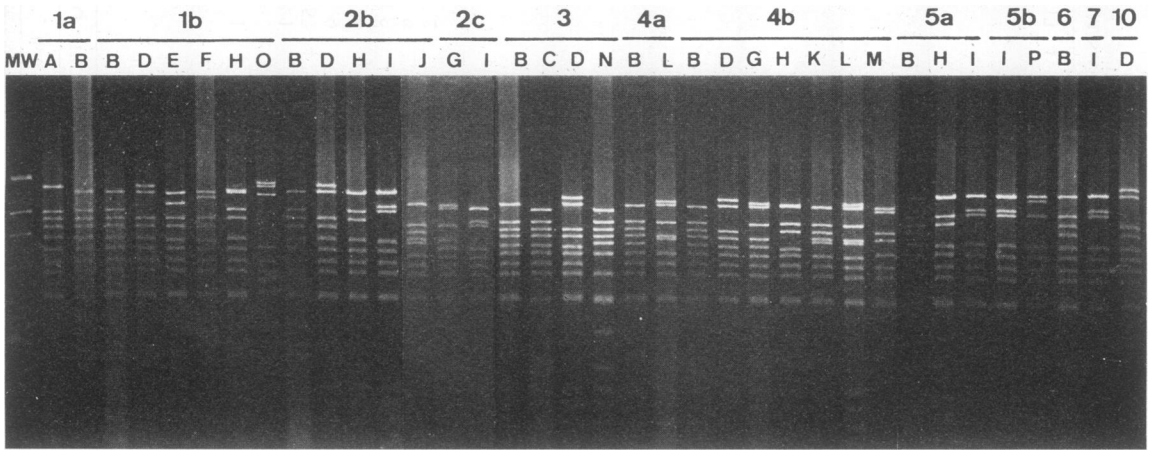

FIG. 1. REAP patterns of *Bam*HI digestion of plasmid DNA within each serotype of *Y. pseudotuberculosis*. Lane MW, lambda DNA digested with *Hin*dIII as a molecular weight marker. Each strain of *Y. pseudotuberculosis* is given in Table 2.

TABLE 3. Epidemiological survey of *Y. pseudotuberculosis* infections

Type of infection	Area	No. of cases	Serotype	REAP pattern	No. of isolates from:										
					Humans	Spaghetti	Kitchen	Vegetables	Well water	Stream water	Soil	Sand	Cats	Wild mice	Deer
Familial outbreak	Shimane, Japan	1	1b	D	2						1	1	1		
			3	B	1						1	1	1		
Outbreak	Primorsk, Russia	1	4b	D	15	1	2	3						1	
Sporadic cases	Shimane, Japan	1	4b	G	1									1	2
			4b	L						1					4
	Okayama, Japan	2	2b	I	2				2						
		1	2c	I	1				1						
		3	4b	G	4				3						
		3	5a	I	3				3						
		9	5b	I	9				9						

unclear. The possibility of transmission of *Y. pseudotuberculosis* to humans through beef and milk deserves attention because some strains of *Y. pseudotuberculosis* have been isolated from cattle (8, 14).

The contamination of environmental substances such as soil and water with *Y. pseudotuberculosis* has been reported by European and Japanese investigators (1, 3). Our recent survey of wild animals (4) and mountainous river water (3) revealed the harboring of *Y. pseudotuberculosis* in wild animals and a high contamination of environmental substances with *Y. pseudotuberculosis*. Almost all the clinical isolates in Japan were obtained from children living in mountainous areas; the families drank untreated drinking water from mountain streams or wells (5, 13). Most of the REAP patterns of human isolates were observed among many isolates from wild animals and well and river water samples (Table 3). This close molecular relation among isolates from wild animals, environmental substances, and patients is demonstrated for the first time, and these findings suggest that wild animals are an important reservoir for *Y. pseudotuberculosis* and that *Y. pseudotuberculosis* is transmitted to humans through environmental substances contaminated by wild animals carrying this species.

Data on an epidemiological survey of actual cases of infection are shown in Table 3. In an outbreak in Primorsk, Vladivostok, Russia, in 1985, the identical REAP pattern of serotype 4b was observed among isolates from patients, food, vegetables (potatoes, onions, and beets), the kitchen table and utensils, and rodent feces found in the kitchen. In a sporadic case in a valley area of Shimane, Japan (6), the potential for transmission of serotype 4b through water contaminated by wild animals carrying this species was shown. REAP of isolates in sporadic human infections examined in Okayama, Japan, showed identical REAP patterns between clinical human isolates and isolates from well water which had been drunk by the patients. All this evidence taken together supports our hypothesis.

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