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Molecular evidence of *Plesiomonas shigelloides* as a possible zoonotic agent

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Abstract The most frequently used method for establishing epidemiological relationships between *Plesiomonas shigelloides* strains is O:H serotyping. However, a number of strains are not serotypeable and isolates from diverse sources can display the same serovar. Moreover, since the zoonotic nature of *Plesiomonas* has been suggested and this hypothesis is based on the identical serovars found in animals and humans, we intend to use four DNA-based techniques: random amplified polymorphic DNA-PCR, enterobacterial repetitive intergenic consensus-PCR, repetitive extragenic palindromic-PCR, and pulsed field gel electrophoresis in order to screen 24 strains belonging to nine O:H serovars isolated from humans, animals, and the environment. In general, *P. shigelloides* showed a high genetic heterogeneity. Three pairs of strains, each containing a human and an animal isolate, displayed similar genotypes. This is the first report that provides molecular evidence that *P. shigelloides* may be zoonotic.

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Plesiomonas shigelloides, a new member of the Enterobacteriaceae family (Garrity et al. 2001), is regarded as an emerging enteric pathogen. It is implicated in both intestinal and extra-intestinal infections in humans (Vandepitte et al. 1980; Rautelin et al. 1995; Lee et al. 1996). In addition, there are also reports on plesiomonad involvement in cases of diarrhea in animals (Foster et al. 2000; Jagger et al. 2000; Niskanen and Salmela 2000). These microorganisms can be found in most aquatic environments (Zakhariev 1971; de Mondino et al. 1995; Krovacek et al. 2000). They have also been isolated from a wide range of different animals, such as shellfish, snakes, freshwater fish, goats, swine, cats, dogs, and monkeys (Davis et al. 1978; Arai et al. 1980; Rutala et al. 1982; Bardon 1999; González et al. 1999). The bacteria have also been detected in fresh vegetables in Costa Rica (Monge et al. 1998). The role of *P. shigelloides* as a zoonotic agent has been hypothesized, although no definitive molecular evidence for this has yet been presented (Davis et al. 1978; Arai et al. 1980).

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Serotyping is, so far, the method of choice for typing strains of *P. shigelloides*. Two major schemes based on somatic (O) and flagellar (H) antigens were first developed by Shimada and Sakazaki (1978), further improved by Aldova (1994) and Aldova and Schubert (1996). Only a few antigens can be correlated with a specific geographical region and/or the source of isolation (Aldova 1994, 1997; Bravo et al. 2000). New methods for typing bacteria at a deeper level have been developed during recent years. Random amplified polymorphic DNA (RAPD)-PCR, enterobacterial repetitive intergenic consensus (ERIC)-PCR, repetitive extragenic palindromic (REP)-PCR, and pulsed field gel electrophoresis (PFGE) are examples of the available molecular weaponry for genotyping of bacteria (Olive and Bean 1999). Various techniques capable of detecting differences at the genetic level have shown greater or lesser abilities relating to the differentiation of phenotypically identical strains (Chatellier et al. 1999; Petersen et al. 2001; Welinder-Olsson et al. 2002). However, knowledge of the genomic diversity of plesiomonads is still very limited. This study was undertaken to examine the relationship between *P. shigelloides* strains that share serotypic data. Genetic differences were analyzed by the four DNA-based techniques mentioned. We provide here evidence that plesiomonads might be involved in cases of zoonosis.

Materials and methods

Bacterial strains and serotyping

Twenty-four strains belonging to nine O:H serovars and isolated from humans (n=10), animals n=11), and environmental origins (n=3) were studied (Table 1). The strains were selected from our laboratory of *P. shigelloides* collection. The criterion for selection was that at least two strains of the same serovar should be available. Moreover, one strain isolated from an animal (dog) in Finland with unknown O antigen (ONT:H3) and another strain isolated in the same country from a human with O66:H2 serovar

Table 1 *P. shigelloides* strains

No. in gel	Strain	Serovar	ERIC	REP	RAPD	PFGE	Country	Origin
1	520/91	O66:H3	1	1	1	1	F	*Bucephala clangula*
2	397/95	O66:H3	2	2	2	2	F	*Lepus timidus*
3	660/97	O66:H3	2	3	3	3	F	*B. clangula*
4	B1129/2000	O66:H3	2	4	4	4	F	*Botaurus stellaris*
5	IH 41386	O66:H3	3	5	5	5	F	Fish (UDS)[b]
6	19A	O66:H3	4	6	6	6	S	Environment
7	32/120	O66:H3	5	7	7	7	SR	Environment
8	B1567/2000	O (UK)[a]:H3	6	8	8	8	F	*Canis familiaris*
9	IH 96003	O66:H2	7	9	9	9	F	Human
10	241/97	O22:H3	8	10	10	10	F	*Felis catus*
11	2044	O22:H3	9	11	11	11	C	Human
12	CAT12	O22:H3	9	11	11	11	C	*F. catus*
13	IH 40845	O22:H3	10	7	12	12	F	Human
14	11A	O22:H3	11	12	13	13	S	Environment
15	IH 40904	O40:H6	12	13	14	14	F	Human
16	IH 40936	O40:H6	13	14	15	15	F	Human
17	IH 41154	O40:H6	13	15	16	16	F	Human
18	IH 111439	O90:H6	13	16	17	17	F	Human
19	878/90	O90:H6	13	16	17	18	F	*Vulpes vulpes*
20	2416	O11:H2	14	17	18	19	C	Human
21	CAT3	O11:H2	14	17	18	19	C	*F. catus*
22	1642	O35:H11	15	13	19	20	C	Human
23	CAT19	O35:H11	16	18	20	21	C	*F. catus*
24	29480	O27:H8	17	19	21	22	CR	Human

C Cuba, *CR* Czech Republic, *F* Finland, *S* Sweden, *SR* Slovak Republic

[a] Unknown O serovar

[b] Undetermined species

were included. A *P. shigelloides* strain, no. 29480 (obtained from the National Culture Collection, Prague, Czech Republic and kindly provided by Dr. E. Aldova), was used as the reference strain. All strains were biochemically characterized by the API 20 system (bioMérieux SA, France) and serotyped as described below. The serological typing was performed according to the schema of Shimada and Sakazaki (1978), Aldova (1994), and Aldova and Schubert (1996) using specific *Plesiomonas* antisera kindly provided by Dr. Aldova. Furthermore, all the strains used in this study have been previously confirmed as *P. shigelloides* using a specific *Plesiomonas* PCR based on the 23S rRNA gene (González-Rey et al. 2000).

DNA isolation for RAPD-, ERIC-, and REP-PCR

DNA was obtained essentially as described by Wilson (1994). The CTAB–protein/polysaccharide complex formed during the process was removed by phenol–chloroform extraction. Concentration and purity of the prepared DNA was measured by the Gene Quant system (Pharmacia Co. Ltd., UK).

RAPD polymerase chain reaction

In this study, we used the "RAPD analysis beads" kit (Pharmacia Biotech, Sweden). We tested the six different primers included in the kit (data not shown). The primer "number 1," with the sequence GGTGCGGGAA (G+C content of 70.2%), was selected for further study because of the largest number of amplicons yielded and the best differential pattern among all the primers checked (data not shown). The PCR was carried out following the manufacturer's protocol (Pharmacia Biotech). Twenty-five picomoles of primer "number 1" and 100 ng of template DNA were added to the analysis bead that contains thermostable polymerases (Amplitaq and Stoffel fragment), bovine serum albumin, and buffer components. The final volume was adjusted to 25 μL by the addition of distilled water. PCR amplifications were performed using a thermocycler (Geneamp PCR System 2400, Perkin Elmer Corp., USA). The PCR profile consisted of initial DNA denaturation (95°C for 5 min) followed by 45 cycles of subsequent denaturation (95°C for 1 min), annealing (36°C for 1 min), and extension (72°C for 2 min). After thermal cycling, the amplified DNA fragments were electrophoresed in a 1.5% agarose gel (Nusieve 3:1 agarose, FMC Bioproducts, USA) in standard Tris-borate buffer (89 mmol/L Tris-borate, 2 mmol/L EDTA). The gel was then stained in distilled water containing ethidium bromide (1 mg/mL) for 30 min. A Mixed Ladder marker of 100, 200, 300, 400, 5,000, 600, 700, 800, 900, 1,000, 2,000 and 3,000 bp (Invitrogen, the Netherlands) was included in the gels for molecular size standards. The patterns of DNA fragments were recorded using UV light (Ultraviolet transilluminator, UVP, USA) and the "ImageStore 5000 Annotator" system (Ultra Violet Products Ltd., UK).

ERIC- and REP-PCR

The repetitive sequence PCR was basically performed following the method of Versalovic et al. (1991). The primers were: ERIC2 (AAGTAAGTGACTGGGGTGAGCG) for the ERIC-PCR, and REP1R-I (IIIICGICGICATCIGGC) and REP2-I (ICGICTTATCIGGCCTAC) for the REP-PCR. The PCR cycling profiles were: 95°C for 5 min followed by 35 cycles of 95°C for 2 min, 40°C for 1 min for REP- or 52°C for 1 min for ERIC-PCR and 72°C for 1 min, with a final single extension at 72°C for 10 min. The PCR products were electrophoresed, stained, and recorded as described above. The molecular size standards were the same as in RAPD-PCR.

Pulsed field gel electrophoresis

PFGE was essentially carried out as in an earlier work by Maslow et al. (1993). Briefly, bacteria were streaked onto Luria–Bertani agar (Sigma-Aldrich, Sweden) and incubated overnight at 37°C. Cells were suspended in 10 mmol/L Tris, pH 8.0, containing 1 mol/L NaCl, 200 mmol/L EDTA, 0.5% Sarcosyl, and 0.2% natrium deoxycholate (EC buffer) and mixed with 350 μL of low melting agarose (Amersham Pharmacia, Sweden) and 35 μL of lysozyme (Roche Diagnostics Scandinavia AB, Sweden). After cooling, the bacteria embedded in the plugs were lysed in a solution containing 2.5 mL of EC buffer and 85 μL of proteinase K (10 mg/mL; Roche Diagnostics Scandinavia AB) and incubated for 24 h at 56°C. Agarose plugs were washed once with 10 mmol/L Tris, pH 8.0, containing 1 mmol/L EDTA (TE buffer) added to tubes containing 5 mL of TE buffer and 50 μL of 20 mmol/L Pefablock (Roche Diagnostics Scandinavia AB) and incubated for 2 h at 37°C. Thereafter, the plugs were washed with the same buffer (2×30 min at 37°C). The DNA in agarose was cut with 10 U of *Spe*I (MBI Fermentas, Germany) overnight at 37°C. The CHEF-DR III variable-angle pulsed field electrophoresis system (Bio-Rad Laboratories, CA) was used for the electrophoresis. The program consisted of two phases, both run at 14°C: the first phase was performed for 19 h at 6 V/cm with a 1- to 20-s linear ramp time and the second phase carried out for 3 h at 6 V/cm with a 20- to 25-s linear ramp time. The gels were stained with ethidium bromide and photographed under UV light. A Lambda Ladder PFG Marker of 48.5, 97.0, 145.5, 194.0, 242.5, 291.0, 339.5, 388.0, 436.5, 485.0, 533.5, 582.0, 630.5, 679.0, 727.5, 776.0, 824.5, 873.0, 921.5, 970.0, 1,018.5 kb (New England

BioLabs, MA) was included in the gels for molecular standard.

Comparing the genomic profiles

The banding patterns obtained by the different genotyping methods were compared for similarity by visual inspection. They were considered different if the presence or absence of at least one band varied in one of the patterns. Nevertheless, we followed Tenover's criteria for interpreting PFGE patterns (Tenover et al. 1995). The different profiles within each method were numbered consecutively starting from 1. Alterations in band intensities were not contemplated as differences.

Results

ERIC polymerase chain reaction

The number of ERIC-PCR bands from the individual ERIC2 primer ranged from 4 to 9, with molecular sizes of 0.1 to >2.0 kb (Fig. 1a). ERIC-PCR divided the 24 strains into 17 genotypes (Fig. 1a). Three of the seven O66:H3, two of five O22:H3, two of three O40:H6 strains, and two of two O90:H6 as well as O11:H2 strains were genotypically identical within each serotype. In addition, the identical genotype (profile ERIC-13) was found among strains of O40:H6 and O11:H2.

REP polymerase chain reaction

The number of REP-PCR bands from the REP1R-I and REP2-I primers ranged from 5 to 12, with molecular sizes of 0.1 to >2.0 kb (Fig. 1b). REP-PCR divided the 24 strains into 19 genotypes (Fig. 1b). Two of the five O22:H3 strains and two of two O90:H6 as well as O11:H2 strains presented the same genotype. Furthermore, the profile REP-13 was found among strains of O40:H6 and O35:H11.

RAPD polymerase chain reaction

The number of REP-PCR bands from primer "number 1" ranged from 3 to 9, with molecular sizes of 0.1 to >2.0 kb (Fig. 1c). RAPD divided the 24 strains into 21 genotypes (Fig. 1c). Two of the five O22:H3 strains and two of two O90:H6 and O11:H2 showed identical genotype.

Pulsed field gel electrophoresis

PFGE gave the highest number of genotypes, dividing the 24 strains into 22 types (Fig. 2). Two of five O22:H3 strains and two of two O11:H2 displayed the same genotype.

Discussion

Recognition of *P. shigelloides* as a human enteric pathogen (Stock 2004) as well as on the pathogenic potential of this

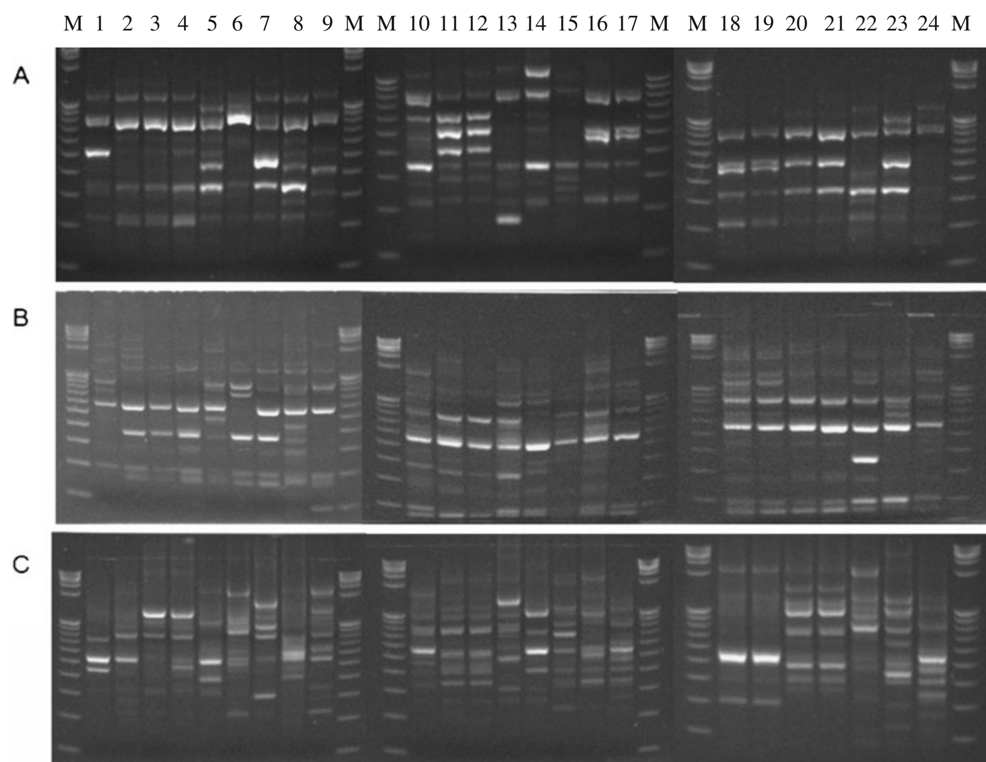

Fig. 1 ERIC-PCR profiles (**a**), REP-PCR profiles (**b**), RAPD profiles (**c**). Strains (from *left* to *right*): 520/91, 397/95, 660/97, B1129/2000, IH 41386, 19A, 32/120, B1567/2000, IH 96003, 241/97, 2044, CAT12, IH 40845, 11A, IH 40904, IH 40936, IH 41154, IH 111439, 878/90, 2416, CAT3, 1642, CAT19, 29480. *M* molecular weight marker ("Mixed Ladder," Invitrogen)

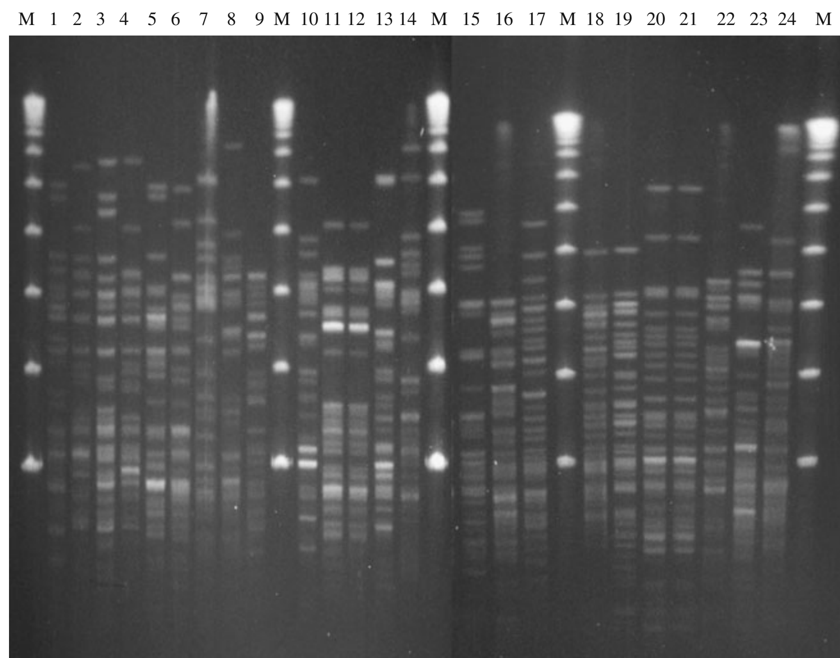

Fig. 2 PFGE profiles. Strains (*from* left to *right*): 520/91, 397/95, 660/97, B1129/2000, IH 41386, 19A, 32/120, B1567/2000, IH 96003, 241/97, 2044, CAT12, IH 40845, 11A, IH 40904, IH 40936, IH 41154, IH 111439, 878/90, 2416, CAT3, 1642, CAT19, 29480. *M* molecular weight marker ("Lambda Ladder PFG Marker," New England BioLabs)

species (Salerno et al. 2010) has increased during the last years. In a region of Japan, *P. shigelloides* ranks first (66.7%) in the list of bacteria isolated in cases of traveler's diarrhea, ahead of other bacteria genera such as *Vibrio*, *Aeromonas*, *Shigella*, and *Salmonella* (Ueda et al. 1999). Regrettably, however, information on plesiomonads in temperate regions is scarce. Enteric *P. shigelloides* infections in Finnish patients have been documented (Rautelin et al. 1995), and in the same country, this microorganism has been recovered from various animal species (Niskanen and Salmela 2000). We have isolated and characterized these bacteria from lakes and river water in the central part of Sweden (Krovacek et al. 2000) and even from a lake above the Polar Circle (González-Rey et al. 2003).

Suggestions regarding the zoonotic potential of *Plesiomonas* appeared as early as 1978. Possible transmission of *P. shigelloides* from a snake to human was discussed by Davis et al. (1978). Two years later, Arai et al. (1980), based on serovar information from isolates from cats, dogs, and humans, concluded that the animals may be important carriers of these microorganisms and therefore play a role in human infections. However, a following analogous study by Aldova (1994), in which *P. shigelloides* collected from a patient with diarrhea and her cat were found to represent two different serovars, was not supportive of the hypothesis of the zoonotic nature of these bacteria. To date, no convincing survey of the potential zoonosis has been made and molecular analysis data are absent.

The present study inspects how molecular-based techniques can distinguish between strains of the same serovar. The four methods used performed essentially similarly, although PFGE showed the highest discriminatory ability. The results also demonstrate that some strains isolated from human and animal sources from the same geographical area display similar profiles, which suggests a clonal relationship.

Many investigations have been performed to elucidate clonal relations between phenotypically identical bacterial strains. Biochemical tests, antibiotic resistance profiles, serotyping, phage typing, and multilocus enzyme electrophoresis are the most common tools currently used in clinical laboratories for phenotypic characterization. However, these methods are either not reliable for discriminating between isolates within species or limited to a few reference laboratories. Techniques based on DNA sequence, rather than expression of genes coded by DNA, can discriminate at a deeper level. Besides, they minimize the problems of typeability and reproducibility. RAPD-, REP-, and ERIC-PCR, as well as PFGE, have been utilized in a large number of studies in molecular epidemiology (Olive and Bean 1999). In contrast, there has been considerably less work done using such approach regarding *P. shigelloides* infections. To our knowledge, this paper is the first report on the use of DNA-based procedures for comparative differentiation of strains belonging to the same serovar. Previously, PFGE was employed in an epidemiological study of diarrheal cases due to these bacteria (Shigematsu et al. 2000). The authors could not identify any repetitive pattern that might indicate a common clone. They suggested that a wide variety of clones have the ability to cause illness. In accord with that work, our results reflect a high genetic diversity in plesiomonad population. The data obtained ensure that strains within the same serovar can generally be differentiated at a genomic level. RAPD has also been used for genotyping *P.*

shigelloides isolates from fish, human clinical sources, and fresh water (Gu et al. 2006). Final conclusions were the same as in the above work: *P. shigelloides* shows a significant level of genetic variability among the isolates.

A major result of this work is that we have found two pairs of strains, each of which consisting of one of human and one of animal origin, to be identical (ERIC/REP/RAPD/PFGE 2044 and CAT12; ERIC/REP/RAPD/PFGE 2416 and CAT3) or, a third one, highly similar [PFGE pattern numbers 17 (IH111439) and 18 (878/90)]. Notably, the counterparts originated from the same country. According to Tenover et al. (1995), two to three fragment differences between isolates can be interpreted as belonging to the same outbreak. The relatedness implied by the analysis of the PFGE typing profiles was in agreement with the results of ERIC-, REP-, and RAPD-PCR for the same strains. Although it is known that following contact with contaminated water and/or food *P. shigelloides* can infect humans and animals, the role that pets or wild animals play in the spread of this microorganism is still unclear. Whether animals and humans were infected independently with genetically identical organisms via direct contact with water and/or contaminated food or that the animals were the source of the human infection is a difficult issue to determine. However, the present study confirms that it is possible for isolates with the same genetic profile to infect both animals and humans.

The findings of the present study underline a need for the molecular comparison of strains isolated from outbreaks and those recovered from the environments where these outbreaks occur. Unfortunately, only few outbreaks due to *P. shigelloides* are reported, which makes obtaining respective bacteria isolates more difficult. Further research on elucidating the manner in which pets and wild animals are infected and how this is related to infections in humans should gain increased attention.

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