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# An epidemiological study of *Plesiomonas shigelloides* diarrhoea among Japanese travellers

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## SUMMARY

*Plesiomonas shigelloides* is often regarded as a non-pathogenic bacterial species that is occasionally isolated from patients with diarrhoea. However, a review of travellers returning to Japan with diarrhoeal illness through Kansai Airport revealed that the incidence of *P. shigelloides* from microbiologically confirmed cases increased from 23·2% in 1987 to 77·8% in 1999. We carried out a descriptive epidemiological study to identify patterns associated with diarrhoea due to this organism. Selected *P. shigelloides* isolates from this patient group were compared by pulsed-field gel electrophoresis of *Spe*I total chromosomal DNA digests to determine their genetic heterogeneity. Over the study period (whole of 1996 and first 2 months of 1999), 1149 of 1659 (69·3%) patients with microbiologically confirmed gastroenteritis yielded *P. shigelloides*. Infection was characterized by watery diarrhoea five times per day that persisted for 3 days. No statistically significant association was found between factors such as age, gender, destination, length of trip, but multivariable logistic regression analysis revealed an association between additional symptoms (vomiting, fever, abdominal pain) age and gender. The molecular fingerprints of a selection of 39 isolates and 3 reference strains of *P. shigelloides* were highly variable and each had a unique profile. We conclude that although *P. shigelloides* infections are usually mild and self-limiting, this organism may contribute to a significant proportion of travellers' diarrhoea in the Orient. The species is characterized by great heterogeneity at the DNA level.

## INTRODUCTION

Travellers' diarrhoea (TD) is defined as any loose stools occurring during travel or as a result of travel [1]. Although 30% of travellers can expect to suffer from TD (10 million episodes per year), 20–45% of all cases remain of undetermined aetiology because of its mild and self-limited nature [2, 3]. These cases are thought to be most likely of bacterial origin as treatment with antimicrobial agents diminishes symptoms [4]. Diarrhoea caused by *Plesiomonas shigelloides* is a typical example of a benign gastrointestinal infection which rarely threatens life. However, its frequency among travellers from developed countries such as Japan in recent times [5] has increased its profile as a public health problem as well as its impact on tourism.

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*P. shigelloides* is a Gram-negative, facultatively anaerobic rod, which is found primarily in freshwater but may also be recovered from river estuaries and seawater, as well as from a variety of fish and shellfish [6–8]. Most cases of *P. shigelloides* diarrhoea in man are sporadic but epidemics have been reported [9, 10] with contaminated food and water being the most common sources. *P. shigelloides* is a rare cause of extraintestinal disease (bacteraemia, meningitis) in neonates but its clinical significance has been questioned [11, 12]. The species has low intrinsic pathogenicity but a number of putative virulence factors have been described [13, 14]. Isolates are usually resistant to most $\beta$ lactams, through a constitutive $\beta$-lactamase, and aminoglycosides [15, 16].

Travellers entering Japan from geographical areas such as Southeast Asia are asked to complete a questionnaire detailing their recent infectious history and, if symptomatic, are expected to submit an appropriate specimen for laboratory analysis. Since 1982, *P. shigelloides* has been listed as a food poisoning organism in Japan. At the Kansai Airport Quarantine Station, the isolation rate of the species from stool samples has increased by approximately threefold since 1987, making it the most frequent bacterial agent isolated from travellers' diarrhoeal samples today [5, 17].

We set out to study factors associated with travellers' diarrhoea that correlated with the presence of *P. shigelloides* in stools by analysing data from questionnaires completed by passengers. In addition, we examined the genetic heterogeneity of selected isolates of the organism by DNA macrorestriction analysis to determine whether certain strains were prevalent in specific locations and/or were associated with severity of disease.

## METHODS

### Study design and data collection

The descriptive epidemiological study was based on ongoing surveillance at Kansai International Airport during the whole of 1996 and 2 months of 1999. Quarantine questionnaire data were kindly provided by the Laboratory Division, Kansai Airport Quarantine Station (Dr K. Kotake). All passengers entering Japan through this airport from Southeast Asia were requested in flight to complete a questionnaire. Assistance was provided for cases of illiteracy and all data were treated confidentially.

Data included identification number (ID), name, age, sex, flight number, travel group size, countries visited, duration of stay, country suspected as the place of infection, date of onset of illness, duration of illness, clinical symptoms, particularly the number of loose stools per day and laboratory confirmation of infecting agents. The working case definition of a diarrhoeal case for this study was any loose stools occurring during travel or as a result of travel.

### Isolation and identification of *P. shigelloides*

Stool samples or rectal swabs were cultured on appropriate media for the isolation of bacterial enteric pathogens [5]. Isolates were classified presumptively as *P. shigelloides* by their production of cytochrome oxidase, motility and fermentation of inositol and mannitol. Forty isolates from the 1149 *P. shigelloides* positive diarrhoeal patients were received for further study and examined for the characteristic morphology of pink colonies with a red edge on Inositol-Brilliant Green-Bile salts (IBB) agar [18]. Species identification was confirmed by API 20E (bioMérieux, Marcy-l'Etoile, France). The characteristic cellular morphology of *P. shigelloides* with multiple flagella was confirmed with negatively stained (0·5%) uranyl formate) preparations in an electron microscope.

Three reference strains, NCTC 10360, NCTC 10363, and NCTC 10364 were obtained from the National Collection of Type Cultures (NCTC), UK.

### Preparation of genomic DNA

Bacterial cells from overnight nutrient agar culture were suspended in SE buffer (75 mM NaCl, 25 mM EDTA $Na_2$,) to an opacity equivalent to McFarland's tube 4, and embedded in 1% (w/v) low-melting-temperature agarose (Sea Plaque agarose, Bio Products, Rockland USA) in SE buffer. Bacteria were lysed at 56 °C on two consecutive nights in buffer (1%, w/v, *N*-lauroyl sarcosine (BDH Laboratory Supplies, Poole, UK), 500 mM EDTA $Na_2$, 500 $\mu$g/ml proteinase K (Sigma Chemical Co., St. Louis, USA) at pH 9·5. The blocks were washed and kept in TE buffer (10 mM Tris–HCl, 10 mM EDTA $Na_2$; pH 7·5) at 4 °C until use [19].

### Pulsed-field gel electrophoresis (PFGE)

DNA in blocks was incubated overnight in reaction buffer prior to overnight digestion with 20 U of *Spe*I (Boehringer–Mannheim GmbH, Mannheim, Ger-

many) at 37 °C. Samples were run in the CHEF DR II apparatus (Bio–Rad, Hemel Hempstead, UK) for 30 h, at 12 °C with 200 V [19]. The pulse-time was ramped from 1 to 30 sec during the run. A concatamer of lambda DNA ladder (Bio–Rad) was used as a standard molecular size marker. The gels were stained for 1 h with ethidium bromide (Sigma) and photographed under UV transillumination. Profiles were saved on computer as 'tif files' for analysis by GelCompar Version 4.1 (Applied Maths, Kortrijk, Belgium) software [19]. Gel data were installed into the GelCompar analysis program through Picture Publisher LE 4.0a (Micrografx Inc., USA) software. Isolates were assessed by the Dice coefficient analysis comparing similarity of each band profile [20] and presented as a dendrogram.

### Statistical analysis

Epi Info, Version 6.04 (CDC, Atlanta, USA) software was used for descriptive and single variable data analysis. The Kruskal–Wallis test was used to analyse the data that were not normally distributed. STATA Release 6.0 (STATA Corporation, Texas, USA) was used for single variable and multivariable analysis.

## RESULTS

### Descriptive epidemiology

During the 14 months of the survey 6638448 travellers passed through Kansai International Airport. The number for the whole of 1996 was 5648066 and for the comparable time periods of 2 months in 1996 and 1999, the numbers were 869321 and 990382 respectively. The numbers of symptomatic cases of diarrhoea for each of these time periods were 9909 (0·18%), 1578 (0·18%) and 2436 (0·25%) respectively.

*P. shigelloides* was isolated from 989 cases of diarrhoea in 1996 (Table 1) and by extrapolation from the ratio of the number of cases in January and February to the annual total, 1198 travellers would have been infected in 1999. There was a slight decrease in the male/female ratio between the 2-monthly samples of each study year and approximately one-fifth of cases were associated with mixed infections. There was no statistical difference between combinations of species recovered together but a trend towards co-isolation of *Aeromonas sobria* (3·8%) or *Vibrio parahaemolyticus* (7·0%) was observed.

In the two study periods, the average age of travellers infected by *P. shigelloides* was 29 years (median 25). The most frequent destinations were Thailand, Indonesia and Vietnam. Subjects most often travelled in pairs for periods of 5 days. The onset of diarrhoea occurred early in the trip especially in younger travellers and tended to end within 2 days. Older subjects began diarrhoea later and suffered longer, but this difference was not statistically significant. Single variable analysis showed a weak association between age group and onset of diarrhoea and age group and duration of diarrhoea. Only 4% of subjects under 15 years of age exhibited diarrhoea after day 6 of travel compared with over 20% of those aged 36 or over. Further, 67% of persons greater than 36 years had diarrhoea for 4 days or longer whereas less than half of the under 15s had diarrhoea for this duration. Multivariable logistic regression analysis revealed significant associations between cases with diarrhoea and additional symptoms (either abdominal pain vomiting or fever), age, and gender. Females tended to have additional symptoms more frequently than males (OR 2·2, 95% CI 1·6–2·8) and older subjects ($\geqslant$ 36 years) had additional symptoms less frequently than the younger age group ($\leqslant$ 15 years) (OR 0·2, 95% CI 0·08–0·77). Travellers in the age range 20–30 years accounted for more than half of all *P. shigelloides* cases and this was associated with a seasonal increase both during summer and end of term holidays (end of February to first week of April for universities in Japan).

### Bacteriological characteristics

The country of origin and patient details for the 40 strains of *P. shigelloides* available for characterization from 1996 and 1999 are shown in Table 2. Biochemical activity in the API 20E distinguished two groups that differed only in $\beta$-galactosidase activity (90% positive and 10% negative). All strains produced cytochrome oxidase, fermented glucose, inositol and mellobiose with acid but without gas production. Production of arginine dihydrolase and lysine and ornithine decarboxylases was uniform. Tests for sucrose fermentation, urease, gelatinase and $H_2S$ production were negative [21]. Cells were actively motile with a rod-shape of 0·8 $\mu$m by 2·8 $\mu$m with rounded ends. Lateral flagella were not clearly visible, but 2–5 polar flagella (2–3 times the length of the cell) were observed.

### PFGE profile and phylogenetic analysis

Preliminary experiments with *Not*I, *Xba*I, *Bgl*II, *Pac*I and *Spe*I on selected strains showed that the latter

Table 1. *Statistical data for overseas travellers with diarrhoea due to* Plesiomonas shigelloides *at Kansai International Airport*

| Year | 1996 | 1996 (Jan., Feb.) | 1999 (Jan., Feb.) | Total |
|---|---|---|---|---|
| Total cases | 989 | 132 | 160 | 1149 |
| Male | 553 | 75 | 75 | 628 |
| Female | 436 | 57 | 85 | 521 |
| Male:female | 1·3:1·0 | 1·3:1·0 | 0·9:1·0 | 1·2:1·0 |
| Mixed infection (%) | 206 (20·8) | 23 (17·4) | 29 (18·1) | 235 (20·5) |
| Day of onset in trip | | | | |
| Mean (SE*)/median (LQ, UQ†) (days) | 2·5 (0·13)/2 (1, 3) | 2·4 (0·26)/2 (1, 3) | 2·1 (0·17)/2 (1, 4) | 2·5 (0·11)/2 (1, 3) |
| Frequency of diarrhoea | | | | |
| Mean (SE*)/median (LQ, UQ†) (times/day) | 4·3 (0·10)/4 (3, 5) | 4·1 (0·20)/3 (2, 5) | 5·2 (0·30)/4·5 (2·5, 6) | 4·5 (0·10)/4 (3, 6) |
| Duration of diarrhoea | | | | |
| Mean (SE*)/median (LQ, UQ†) (days) | 3·2 (0·12)/2 (2, 4) | 2·9 (0·24)/2 (2, 3) | 3·1 (0·17)/2 (2, 4) | 3·2 (0·11)/2 (2, 4) |
| Number of members in a travel group median (LQ, UQ†) | 2 (2, 4) | 2 (2, 4) | 2 (2, 5) | 2 (2, 4) |
| Age profile of cases | | | | |
| Mean/median/range (years) | 29/25/3, 76 | 29/25/12, 70 | 29/25/13, 76 | 29/25/3, 76 |
| Age group composition | | | | |
| Number (%) | | | | |
| Group 1 ($>15$) | 23 (2·3) | 2 (1·5) | 2 (1·3) | 25 (2·2) |
| Group 2 ($\geqslant 15$, $<20$) | 63 (6·4) | 4 (3·0) | 6 (3·8) | 69 (6·0) |
| Group 3 ($\geqslant 20$, $<25$) | 367 (37·1) | 54 (40·9) | 70 (43·8) | 437 (38·0) |
| Group 4 ($\geqslant 25$, $<30$) | 231 (23·4) | 33 (25·0) | 36 (22·5) | 267 (23·2) |
| Group 5 ($\geqslant 30$, $<35$) | 106 (10·7) | 12 (9·1) | 18 (11·3) | 124 (10·8) |
| Group 6 ($\geqslant 35$, $<40$) | 46 (4·7) | 7 (5·3) | 6 (3·8) | 52 (4·5) |
| Group 7 ($\geqslant 40$, $<45$) | 36 (3·6) | 6 (4·5) | 3 (1·9) | 39 (3·4) |
| Group 8 ($\geqslant 45$, $<50$) | 39 (3·9) | 4 (3·0) | 5 (3·1) | 44 (3·8) |
| Group 9 ($\geqslant 50$) | 78 (7·9) | 10 (7·6) | 14 (8·8) | 92 (8·0) |
| Major countries of travel‡ (%) | THA (34·8)<br>INA (25·4)<br>VIE (10·9) | THA (43·2)<br>INA (38·6)<br>VIE (5·3) | THA (45·3)<br>INA (29·8)<br>VIE (8·7) | THA (36·3)<br>INA (26·0)<br>VIE (10·6) |

* SE, Standard error.

† LQ, 25th percentile; UQ, 75th percentile.

‡ THA, Thailand; INA, Indonesia; VIE, Vietnam.

Table 2. *Characteristics and background of* Plesiomonas shigelloides *isolates studied by PFGE*

| Isolate identification number | Age | Gender* | Country of infection† | Presence of other symptom‡ | Group size | Onset day of travel | Frequency of diarrhoea | Date of isolation |
|---|---|---|---|---|---|---|---|---|
| KIX-1078 | 5 | F | VIE | − | 2 | 3 | 4 | 01/1996 |
| KIX-1080 | 25 | F | THA | + | n.d.§ | 3 | 3 | 01/1996 |
| KIX-1081 | 32 | F | INA | + | 2 | 2 | 10 | 01/1996 |
| KIX-1083 | 38 | M | INA | − | 8 | 1 | 5 | 01/1996 |
| KIX-1085 | 38 | F | INA | + | 2 | 3 | 10 | 01/1996 |
| KIX-1087 | 12 | M | THA | + | 6 | 0 | 5 | 01/1996 |
| KIX-1088 | 25 | M | THA | + | n.d.§ | 1 | 10 | 01/1996 |
| KIX-1094 | 27 | M | INA | − | 2 | 12 | 3 | 01/1996 |
| KIX-1096 | 70 | M | INA | − | 3 | 2 | 8 | 01/1996 |
| KIX-1107 | 27 | F | INA | + | 2 | 1 | 1 | 01/1996 |
| KIX-1112 | 25 | F | VIE | + | n.d.§ | 3 | 5 | 01/1996 |
| KIX-1114 | 23 | M | THA | + | 40 | 3 | 4 | 01/1996 |
| KIX-1117 | 22 | F | THA | + | 2 | 0 | 2 | 01/1996 |
| KIX-1118 | 37 | F | THA | + | 24 | 0 | 1 | 01/1996 |
| KIX-1119 | 27 | M | THA | + | 4 | 1 | 5 | 01/1996 |
| KIX-1120 | 40 | M | PHI | + | 3 | 25 | 3 | 01/1996 |
| KIX-1121 | 54 | F | THA | + | 3 | 1 | 3 | 01/1996 |
| KIX-1122 | 24 | F | THA | + | 1 | 4 | 3 | 01/1996 |
| KIX-1126 | 30 | M | THA | + | 10 | 3 | 2 | 01/1996 |
| KIX-1129 | 23 | F | THA | + | 3 | 2 | 4 | 01/1996 |
| KIX-1131 | 24 | M | INA | + | 3 | 3 | 6 | 01/1996 |
| KIX-1133 | 39 | F | THA | + | 2 | 3 | 3 | 01/1996 |
| KIX-1136 | 39 | M | THA | + | 1 | 1 | 3 | 01/1996 |
| KIX-1137 | 28 | M | INA | − | 2 | 0 | 3 | 01/1996 |
| KIX-3607 | 58 | F | THA | − | 1 | 3 | 12 | 01/1999 |
| KIX-3611 | 23 | F | INA | + | 2 | 1 | 4 | 01/1999 |
| KIX-3615 | 24 | F | THA | + | 1 | 11 | 2 | 01/1999 |
| KIX-3619 | 29 | F | THA | − | 2 | 0 | 10 | 01/1999 |
| KIX-3620 | 39 | M | MAL | − | 70 | 0 | 2 | 01/1999 |
| KIX-3655 | 20 | F | THA | + | 2 | 0 | 3 | 02/1999 |
| KIX-3664 | 22 | M | INA | + | 2 | 1 | 2 | 02/1999 |
| KIX-3666 | 22 | M | THA | − | 5 | 1 | 2 | 02/1999 |
| KIX-3673 | 26 | F | PHI | + | 2 | 0 | 2 | 02/1999 |
| KIX-3678 | 46 | M | VIE | + | 1 | 1 | 8 | 02/1999 |
| KIX-3684 | 24 | F | INA | − | 3 | 5 | 4 | 02/1999 |
| KIX-3687 | 76 | M | MYA | − | 23 | 5 | 15 | 02/1999 |
| KIX-3692 | 32 | F | THA | + | n.d.§ | 1 | 20 | 02/1999 |
| KIX-3695 | 22 | F | INA | + | 5 | 1 | 10 | 02/1999 |
| KIX-3700 | 22 | M | INA | + | 9 | 3 | 10 | 02/1999 |
| KIX-3703 | 52 | M | CAM | − | n.d.§ | 2 | 10 | 02/1999 |
| NCTC 10360 | | | | | | | | 1954 |
| NCTC 10363 | | | | | | | | 1964 |
| NCTC 10364 | | | | | | | | 1964 |

* M, Male; F, Female.
† THA, Thailand; INA, Indonesia; VIE, Vietnam; PHI, Phillippines; MYA, Myanmar (Burma); CAM, Cambodia.
‡ Abdominal pain, fever, or vomiting.
§ n.d., no data available.

enzyme produced the clearest patterns with 15–25 resolvable bands within a size range of 48·5–291 kb. Due to the relative small size of the DNA fragments, efficient separation was achieved with a pulse time gradient of 1–30 sec for *Spe*I digests. Banding patterns of duplicate digests of the same strain within a gel were identical. One strain did not produce a clear banding pattern even after formalin treatment for the

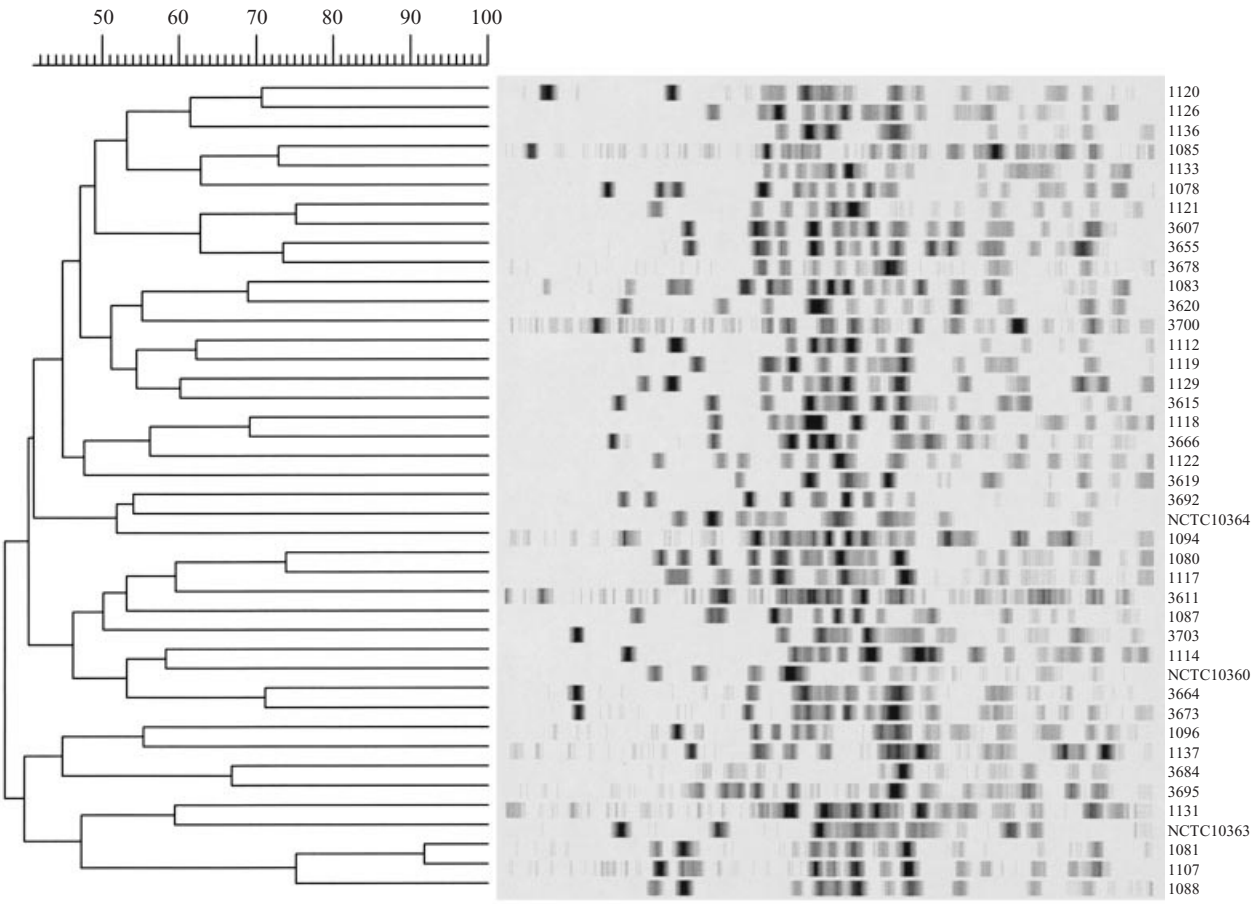


**Fig. 1.** Diversity of *Spe*I digests of chromosomal DNA of *Plesiomonas shigelloides* isolates displayed as a dendrogram of Dice similarity coefficients. The origin of isolates are given in Table 2.

removal of DNase [22] and was therefore eliminated from further analysis by PFGE.

Figure 1 shows the cluster analysis and DNA banding patterns for the 39 clinical and three reference strains examined. Two strains from travellers to Indonesia, isolated from different tourist groups at different times, showed a Dice similarity coefficient of 91·7%. However, all other strains gave discrete profiles and similarity values below 75%.

## DISCUSSION

Travellers' diarrhoea is usually defined as the passage of three or more unformed stools in 24 h during or shortly after travel but recently an episode of single watery diarrhoea was included in the case definition [3–5]. The most common presentation of TD is acute watery diarrhoea and symptoms often occur early in the voyage, the peak onset being on the third day of travel [2, 23, 24]. For this reason, screening of travellers at quarantine stations is effective for surveillance of TD despite the problems of cooperation of airlines, operating companies and passengers, recall bias, and limited time to complete forms.

The descriptive data for *P. shigelloides* diarrhoeal cases in the whole of 1996 and first 2 months of 1999 at Kansai Airport showed that most individuals experienced diarrhoea by the second day with four motions a day. In less than 1%, symptoms persisted for several weeks. Reports of fever were rare and when present it was usually combined with other symptoms. *P. shigelloides* associated diarrhoea is therefore a mild self-limiting infection of short duration similar to many presentations of TD from which no causative organism is isolated. This may suggest that the actual number of *P. shigelloides* gastrointestinal infections is underestimated.

This descriptive study did not take into account well known risk factors associated with TD, for example, untreated water intake, salad or raw seafood consumption, and ingestion of cut fruit. However it

underlines the high prevalence of travellers in adolescence and early adulthood who tend to indulge in adventurous eating habits in exotic and sometimes in unsanitary areas.

Although *P. shigelloides* strains have been compared with *Aeromonas* spp. for phylogenetic studies using 5S rRNA [25] and the 16S-23S intergenic spacer (ITS) region of the rRNA operon [26], genetic diversity within the species has not, to our knowledge, been addressed. Preliminary studies with primers targetting the 16S-23S ITS region in PCR revealed no heterogeneity amongst the strains studied (data not shown) and so DNA macrorestriction digests and PFGE were utilized. The G+C% ratio of *P. shigelloides* is approximately 51% and so the choice of restriction enzyme was empirical. We screened a number of enzymes in preliminary trials but only *Xba*I (T*CTAGA) and *Spe*I (A*CTAGT) gave resolvable DNA fragments to allow comparison of strains. *Spe*I profiles revealed that all strains, with the possible exception of a pair from Indonesia, were unique. These highly variable chromosomal DNA patterns suggest that the pathogenicity of *P. shigelloides* is not restricted to a prevalent clone and the ability to cause diarrhoeal illness is universal within the species. Furthermore, comparison of the DNA profiles did not disclose conserved areas that might be associated with pathogenicity or a common property.

In conclusion, we have shown that *P. shigelloides* is likely to be a significant contributor to enteric infections in travellers in the Orient. Infection is usually mild, self-limiting and not requiring treatment but a minority of patients experience severe and prolonged symptoms. Molecular analysis of selected strains showed a highly diverse population suggestive of widespread dissemination of an almost limitless number of strains.

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