Studies on *Plesiomonas shigelloides* isolated from different environments

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2003

Acta Universitatis Agriculturae Sueciae

Veterinaria 156

Abstract

González-Rey, C., 2003. *Studies on Plesiomonas shigelloides isolated from different environments*. Doctor's dissertation.
ISSN 1401-6257, ISBN 91-576-6383-1.

Plesiomonas shigelloides is an aquatic microorganism recognised recently as potential human and animal pathogen. Plesiomonads are Gram-negative, motile, non-spore-forming bacilli, facultative anaerobic and oxidase positive. Since 2001, *P. shigelloides* belongs to the family *Enterobacteriaceae*. The primary reservoirs for this bacterium are fresh- and estuarine water, mainly in temperate climates.

Although there were reports on the isolation of *P. shigelloides* from human patients in Sweden, the occurrence of this bacterium in Swedish freshwaters had not been studied yet. The presence of plesiomonads in lakes and rivers in Sweden is reported for the first time. Interestingly, some serovars seem to be geographically correlated.

Some bacterial pathogens have been previously found in extreme climates such as the Polar Circle area. Surprisingly, the investigation of fresh water from lakes north of the Polar Circle in Sweden revealed the presence of *P. shigelloides*. Non-agglutinating isolates were found which suggest the finding of new serovars. Molecular techniques showed that they were genotypically different.

Antimicrobial susceptibility tests showed that *P. shigelloides* presents a natural resistance to β-lactams despite the geographical region from where they were isolated. Furthermore, some strains may carry genes for resistance to tetracyclines.

A correct identification of bacteria is crucial to determine the adequate treatment and for epidemiological studies. *P. shigelloides* is not specified in many clinical laboratories; therefore, a PCR for specific detection of this bacterium was developed. Detection was successful for all the isolates tested despite their serovar, source of isolation or geographical origin. The primers did not amplify genetic material from close related bacteria.

Various molecular techniques were evaluated for genotyping *P. shigelloides* of the same serovar. PFGE and RAPD showed the highest discriminatory skills detecting 22 and 21 genotypes, respectively, among 24 strains. Animal-human pairs from the same geographical area presented an equal genotype. This finding is the first molecular evidence of the possible role of *P. shigelloides* as a zoonotic agent.

In conclusion, this thesis research has contributed significantly to our knowledge of *P. shigelloides* by providing new information on its distribution, its specific detection by PCR, intra-species relationship and its possible relation with zoonotic cases.

Keywords: Plesiomonas shigelloides, distribution, Polar Circle, aquatic environment, serotyping, antibiotic susceptibility, PCR, detection, PFGE, genotyping.

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Dedicated to my parents and the sea that I do love –Mare Nostrum

Mediterraneo

Quizá porque mi niñez
sigue jugando en tu playa
y escondido tras las cañas
duerme mi primer amor,
llevo tu luz y tu olor
por dondequiera que vaya,

y amontonado en tu arena
tengo amor, juegos y penas.

Yo, que en la piel tengo el sabor
amargo del llanto enterno
que han vertido en ti cien pueblos
de Algeciras a Estambul
para que pintes de azul
sus largas noches de invierno.

A fuerza de desventuras,
tu alma es profunda y oscura.

A tus atardeceres rojos
se acostubraron mis ojos
como el recodo al camino...

Soy cantor, soy embustero,
me gusta el juego y el vino,
Tengo alma de marinero...

Qué le voy a hacer, si yo
nací en el Mediterráneo.

Y te acercas, y te vas
después de besar mi aldea.
Jugando con la marea
te vas, pensando en volver.
Eres como una mujer
perfumadita de brea

que se añora y se quiere
que se conoce y se teme.

Ay, si un día para mi mal
viene a buscarme la parca.
Empujad al mar mi barca
con un levante otoñal
y dejad que el temporal
desguace sus alas blancas.

Y a mi enterradme sin duelo
entre la playa y el cielo...

En la ladera de un monte,
más alto que el horizonte.
Quiero tener buena vista.

Mi cuerpo será camino,
le daré verde a los pinos
y amarillo a la genista...

Cerca del mar. Porque yo
nací en el Mediterráneo.

Joan Manuel Serrat

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Appendix

Papers I-V

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I.   Krovacek, K., Eriksson, L.M., González-Rey, C., Rosinsky, J. and Ciznar, I. (2000) Isolation, biochemical and serological characterisation of *Plesiomonas shigelloides* from freshwater in Northern Europe. *Comp. Immunol. Microbiol. Infect. Dis. 23,* 45-51.

II.   González-Rey, C., Svenson, S.B., Eriksson, L.M., Ciznar, I. and Krovacek, K. (2003) Unexpected finding of the "tropical" bacterial pathogen –*Plesiomonas shigelloides*- from lake water north of the Polar Circle. Accepted for publication in *Polar Biology*.

III.   González-Rey, C., Svenson, S.B., Bravo, L., Siitonen, A., Pasquale, V., Dumontet, S., Ciznar, I. and Krovacek, K. (2003) Serotypes and anti-microbial susceptibility of *Plesiomonas shigelloides* isolates from humans, animals and aquatic environments in different countries. Submitted for publication.

IV.   González-Rey, C., Svenson, S.B., Bravo, L., Rosinsky, J., Ciznar, I. and Krovacek, K. (2000) Specific detection of *Plesiomonas shigelloides* isolated from aquatic environments, animals and human diarrhoeal cases by PCR based on 23S rRNA gene. *FEMS Immunol. Med. Microbiol. 29*, 107-113.

V.   González-Rey, C., Siitonen, A., Bravo, L., Ciznar, I., Svenson, S.B. and Krovacek, K. (2003) Molecular techniques for genotyping *Plesiomonas shigelloides* of the same serovar. Submitted for publication.

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Abbreviations

APW	alkaline peptone water
BALB/c	"Bagg albino" inbred mice
bp	base pairs
Caco-2 cells	human colonic tumor cells
CHO cells	Chinese hamster ovary cells
CTAB	hexadecyltrimethyl ammonium bromide
DC	deoxycholate citrate agar
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunoabsorbent assay
ERIC-PCR	enterobacterial repetitive intergenic consensus-PCR
FOS	fructo-oligosaccharides
Hep-2 cells	human carcinoma larynx cells
IBB	inositol brilliant bile salt agar
LT	heat-labile toxin
NaCl	sodium chloride
NT-407 cells	normal embryonic intestinal cells
ONPG	orto-nitrophenyl-beta-D-galatopyranoside
PCR	polymerase chain reaction
PDA	Plesiomonas differential agar
PFGE	pulsed-field gel electrophoresis
RAPD	random amplified polymorphic DNA
REP-PCR	repetitive extragenic palindromic-PCR
SS	Salmonella-shigella agar
ST	heat-stable toxin
TE buffer	tris-EDTA buffer
TSB	tryptic soy broth
TTX	tetrodotoxin
UV light	ultra violet light
Vero cells	kidney cells of the African green (vervet) monkey
XDC	xylose-lysine-deoxycholate agar
Y1 cells	mouse adrenal tumor cells

Studies on *Plesiomonas shigelloides* isolated from different sources

The following is a brief introduction to my thesis on this "unknown bacterium", giving background information for a better understanding of my research.

Historical background and taxonomy

The first reference to these bacteria was in 1947 when Ferguson and Henderson (1947) described a microorganism isolated from faeces of a patient with unknown clinical history. It was called Paracolon C27 and because of its biochemical characteristics defined as related to the family *Enterobacteriaceae*. Schmid *et al*. (1954) proposed four different biotypes based on the differences found in the fermentation of dulcitol, lactose and salicin. Since then, this microorganism has been renamed several times. In 1954, Bader (1954) suggested including these bacteria within the genus *Pseudomonas* with the species name *shigelloides*. In 1959, Sakazaki and Namioka (1959) proposed that this microorganism should be named *michigani* because of the geographical location where Ferguson and Henderson isolated this bacterium. Because of the cytochrome oxidase activity and flagellar morphology the bacterium was moved from the *Pseudomonas* genus and included within the genus *Aeromonas* with the species name *shigelloides* (Ewing *et al*., 1961), within the family *Vibrionaceae*. Based on the recommendations of Habs and Schubert (1962), the bacterium was then transferred into a new genus called *Plesiomonas*, thus naming the species *Plesiomonas shigelloides*. However, in 1963, Sebald and Véron (1963) proposed that this bacillus should be integrated within the genus *Fergusonia*. The bacterium has remained within the family *Vibrionaceae* until molecular studies carried out by Martínez-Murcia *et al*. (1992) and Ruimy *et al*. (1994) indicated that *P. shigelloides* is phylogenetically related to the genus *Proteus*. Furthermore, Huys and Sings (1999) in an evaluation of the amplified fragment length polymorphism (AFLP) technique for genotyping *Aeromonas* spp. found that *P. shigelloides* clearly falls out of the major *Aeromonas* cluster. In the light of these recent findings the genus *Plesiomonas* has been moved to the family *Enterobacteriaceae*, and is the only oxidase-positive member of this family (Garrity et al., 2001). However, the discussion remains open because Ruymi *et al*. (1994) have suggested the creation of a new family, *Plesiomonadaceae*, with one species, *P. shigelloides*.

The habitat

Although the bacterium has been isolated from a variety of substrates, the aquatic environment is the first reservoir of this bacterium. It has been isolated from both

freshwater (rivers, streams, ponds, lakes, etc.) and estuarine (brackish) water (Islam *et al.*, 1991; Schubert & Beichert, 1993; de Mondino *et al.*, 1995; Aldova *et al.*, 1999). Also, Zakhariev (1971), de Mondino *et al.* (1995) and Pasquale and Krovacek (2001) isolated *P. shigelloides* from sea water. In addition, Monge *et al.* (1998) isolated this organism from fresh vegetables in Costa Rica. *P. shigelloides* moves and replicates when the temperature is above 8 C. This fact has given plesiomonads the characteristics of thermophilic bacteria. Other physiochemical characteristics of the environment, such as pH, chlorophyll A, suspended particles and organic material can influence the growth of this microorganism and the success of its isolation (Medema & Schets 1993; Schubert & Pelz 1993). Most of the reports on isolation of plesiomonads are from countries situated in the tropical or subtropical areas. This bacterium has also been called the "Asian" bacteria because of the high incidence of isolations in countries such as Japan and Thailand.

P. shigelloides is not limited to Asia, as recent studies in various African countries show that *P. shigelloides* may play an important role in gastro-enteritis and diarrhoeal cases. Moreover, reports on isolation from freshwater in Central Europe have shown that it is ubiquitous in water within these regions. Krovacek and collaborators are testing the hypothesis that *P. shigelloides* is enzootic to Swedish waters (Krovacek *et al.*, 2000). The most surprising results were obtained when water samples from lakes situated north of the Polar Circle were analysed for the presence of this microorganism (Paper II of this thesis). The occurrence of *P. shigelloides* was confirmed in one of the lakes studied. This fact supports the suggestion that *Plesiomonas* is globally distributed.

P. shigelloides has been also isolated from freshwater recreation places (Medema & Schets 1993). These authors described an outbreak that occurred in Amsterdam in 1990 where nine persons presented gastrointestinal symptoms after having had contact with freshwater at a recreational area.

Water from aquaria can be a reservoir for this bacterium. Aldova *et al.* (1999) found 13 positives out of 23 aquariums (56%) from 8 households. In a clinical case in south-eastern Missouri, stools from a 14-month-old girl presenting watery diarrhoea were investigated for the presence of various pathogens. Results were positive for *P. shigelloides* and negative for *Campylobacter*, *Salmonella*, *Shigella*, *Yersinia*, *Aeromonas* and rotavirus. The report showed that the child could have been bathed after the aquarium water had been poured into the bath tube. Samples from the aquarium water gave a positive result for *P. shigelloides*. Thus, an investigation to estimate the prevalence of *P. shigelloides* in tropical fish tanks was carried out in different locations in Missouri, USA. *P. shigelloides* was isolated from four (22%) of the 18 tanks investigated (Centers for Disease Control, 1989) . In another report from Great Britain, the investigators were also able to isolate plesiomonads from aquarium water (Sanyal *et al.*, 1987).

Culture conditions

The isolation and identification of this bacterium from samples by a clinical laboratory, either from humans or animals, depend on the screening of colonies for oxidase and indole positivity and the appropriate use of selective and differential media. Different agars have been used in order to grow this microorganism (Fig. 1). Enteric agars such as MacConkey (Penn *et al*., 1982; Pitarangsi *et al*., 1982; Huq & Islam 1983; Holmberg & Farmer 1984; Rolston & Hopfer 1984), salmonella-shigella agar (SS) (Tsukamoto *et al.*, 1978; Arai *et al*., 1980; Holmberg & Farmer 1984), modified salmonella-shigella agar (Tsukamoto *et al*., 1978), deoxycholate citrate agar (DC) (Huq & Islam 1983), xylose-lysine-deoxycholate agar (XDC), Hektoen enteric agar (Penn *et al*., 1982; Pitarangsi *et al*., 1982; Rolston & Hopfer 1984), deoxycholate lactose agar (Sakazaki & Balows 1981) and Endo agar (Krovacek *et al*., 2000). These media present diverse challenges for differentiation of *P. shigelloides* from other bacteria species. For instance, MacConkey agar supports the growth of plesiomonads but because the genus contains both lactose positive and negative strains (Von Gravenitz 1985) it can not be used for differential purposes. Schubert (1977) found SS agar as the most favourable medium for the growth of this bacillus but it could not differentiate *P. shigelloides* from other members of the family *Enterobacteriaceae* or *Aeromonas* spp. Therefore, other investigators have tried to formulate media that could overcome these problems. Von Gravenitz and Bucher (1983) recommended the use of inositol brilliant bile salt agar (IBB). This medium can differentiate between *Aeromonas* and *Plesiomonas*: *Aeromonas* appears colourless while *Plesiomonas* shows as whitish to pinkish colonies. In a study by Huq *et al*. (1991) *Plesiomonas* differential agar (PDA) was found to be the best among the media they tested for isolating *P. shigelloides* from water and possibly also from clinical samples containing greater numbers of *Aeromonas* spp. These investigators also found that recovery of *Plesiomonas* from PDA was enhanced by using 42 $^{\circ}$C, although 44 $^{\circ}$C was the optimal temperature at which the colony size difference easily distinguished between *Plesiomonas* and *Aeromonas*.

Enrichment broths are also used for recovery of *Plesiomonas*, mainly from environmental samples. Alkaline peptone water (APW) and tetrathionate broth without iodine are generally used. The latter seems to give better results for the recovery of plesiomonads from water samples and oysters (Freund *et al*., 1988a & 1988b). In addition, Rahim and Kay (1988) found bile peptone broth more effective then APW for recovery of *P. shigelloides* from clinical samples. However, these results are controversial. Van Damme and Vandepitte (1980) reported no increase in isolation rate using tetrathionate broth, with and without iodine, for *P. shigelloides* from freshwater fish.

The use of enrichment media may depend on the numbers and composition of competing flora and the choice of solid medium. Therefore, further studies are needed in order to find out whether an enrichment medium can be really useful.

Brenden *et al.* (1988) tested seven common enteric agars in order to describe the colony appearance and the growth characteristics after subculture for 4 weeks. Freshly subcultured, freezer-stored isolates were inhibited by salmonella-shigella agar, eosin-methylene blue agar and brilliant green agar. Four-week-old cultures maintained in tryptic soy broth (TSB) and subcultured before testing presented a > 10^2 increase on brilliant green agar whereas eosin-methylene blue agar no longer sustained the development of plesiomonads.

Morphology

The macro-morphology of *P. shigelloides* reveals different colonial appearance depending on the selective or differential agar used. Colonies vary from flat, round, 1-2 mm size with smooth edges on blood agar to flat, irregular edge and shape and around 1 mm size when plesiomonads are cultured on deoxycholate agar (Fig. 1).
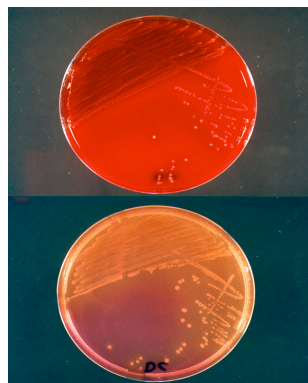

Fig. 1. P. shigelloides colonies on blood (above) and deoxycholate (below) agars.

Micro-morphologically, *P. shigelloides* are Gram-negative, motile, capsulated, flagellated and non-spore-forming bacilli (Fig. 2). The size of a single cell is 0.7-1 m x 2.1-3 m. The distribution, number and shape of the flagella have been used as important criteria for taxonomic studies. However, the fact that there are non-motile strains excludes this character as a reliable property.

The presence of inclusion bodies has been detected at the early stage of growth in *P. shigelloides* by transmission electron microscopy (Pastian & Bromel, 1984). These authors suggested that the granules might be composed of polyphosphates. In a study by Ogawa and Amano (1987), these electron-dense inclusion bodies were also observed. When the authors used light microscopy their results were similar to those obtained by Pastian and Bromel, but when the electron microscopy was used they detected these granules regardless of the bacterial growth phase. An electron microprobe X-ray analysis of the electron-dense inclusion bodies revealed high concentrations of phosphorus and potassium.
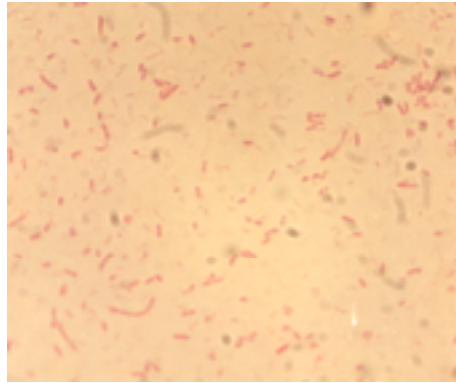
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Fig. 2. Light microphotography of *P. shigelloides* (Gram stain, X 1250).

Magnesium and silicon were detected as well, although the latter was not specific to these granules. Inclusion bodies might play a role in the survival strategies of *P. shigelloides* when the environmental conditions are unfavourable.

Biochemical characteristics

Von Gravenitz (1980) summarised the biochemical characteristics for this microorganism. His scheme is based on the previous studies carried out by Ewing and Hugh (1974). Some minimal requirements for a further characterisation of presumptive *P. shigelloides* colonies are positive reactions for oxidase, indol, inositol, maltose and glucose. However, additional biochemical tests, as shown in Table 1, are always needed for a better identification of this bacillus (Von Gravenitz, 1980). In 1991, Kelly and Kain (1991) added new information about the reactions for ONPG, DNAase, phenylalanine deaminase, motility, lactose- and salicin fermentation. Their results were different from those previously published, the most notable being the DNAase reaction: all the *P. shigelloides* strains they tested were positive. Jeppesen (1995) summarised the differences *between P. shigelloides*, *Aeromonas hydrophila*, *A. caviae* and *A. sobria*. *P. shigelloides* can utilise inositol and ornithine decarboxylase whereas none of the *Aeromonas* spp. was positive for these two assays.

There are a variety of different commercial available kits, such as the TTE-AS and the API 20E, that give reliable results for identification of *P. shigelloides* (Krovacek *et al*., 2000). Finally, the value of biochemical typing is limited due to the phenotypic homogeneity of the species. Minor differences only are observed in carbohydrate fermentation.

Table 1. Biochemical profiles of *P. shigelloides*.

Substrate or property	Reaction
Beta-Galactosidase	+
Arginine Dihydrolase	+
Lysine Decarboxylase	+
Ornithine Decarboxylase	+
Simmons Citrate	-
H_2S	-
Urease	-
Tryptophane deaminase	-
Indole	+
Acetoin (Acetyl Methylcarbinol)	-
Gelatin	-
Glucose	+
Mannitol	-
Inositol	+
Sorbitol	-
Rhamnose	-
Saccharose	-
Melibiose	-
Amygdalin	-
L(+) Arabinose	-
Oxidase	+

Other biochemical characteristics that are not useful in the identification *of P. shigelloides* but important for industrial purposes and for survival strategies are the ability for producing trehalose (Yoshida *et al*., 1998) and chitinase (Ramaiah *et al*., 2000). The toxic effect of mannose on *P. shigelloides* growth has been reported by Rager *et al.* (2000). Mannose 6-phosphate may have a toxic effect for the bacterial cells. Unlike its taxonomic "cousin" *Proteus*, *P. shigelloides* can transport mannose into the cytoplasm, but it cannot be further metabolised because of the lack of the mannose-6-phosphate isomerase. Moreover, Binet *et al.* (1998) found *Aeromonas* spp. capable of utilising mannose as a carbon source. Chitin, a

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complex carbohydrate polymer, is abundant in aquatic environments and forms the cuticle of crustaceans and many molluscs. Like many other aquatic bacteria, *P. shigelloides* can utilise, by means of production of chitinases, this polymeric compound as a carbon source and as a way to penetrate the exoskeleton of different organisms and establish there.

A new restriction endonuclease was found in *P. shigelloides* by Miyahara *et al.* (1990). This enzyme was named *Psh* AI and it can be useful for recombinant DNA technology because of its stability, high yield and novel recognition site (GACNN/NNGTC).

Serology

Unlike other phenotypic methods, serology has more successfully been used for distinguishing different strains of *P. shigelloides*. There are mainly two major serotyping schemes, which are based on somatic (O) and flagellar (H) antigens. One of the antigen schemas was developed by Shimada and Sakazaki (1978) and consisted of 40 serovars. Since 1964, Aldova has been studying the antigenic structure of this organism and in 1968 Aldova & Geizer (1968) identified 13 O- and 15 H-antigens. Since then, new serovars have been added from both schemas until, finally, an international unified scheme was created by the incorporation of the Shimada-Sakazaki and Aldova schemas. According to Aldova (1994), some serotypes are ubiquitous, whereas others are rare and isolated only in certain regions. At the present moment, 102 somatic antigens and 51 flagellar antigens have been recognised (Aldova & Shimada, 2000).

However, most of the strains used in the above studies are originally from clinical specimens of human and homeothermic animal sources. Because *P. shigelloides* is found in water and animals that live in the aquatic environments, Schubert & Pelz (1993) elaborated another antigen scheme in Germany. The strains used for the "Schubert" scheme, which consisted of 23 O- and 5 H-antigens, were isolated from water of ponds and water insects in Germany. Nevertheless, this "environmental" scheme has not been incorporated into the international one. Further studies are needed to overcome this (Aldova & Schubert, 1996).

Sack et al. (1994) hypothesised that, in developing countries, people might be "naturally" immunised against shigellosis by drinking water *containing P. shigelloides*. The reason for such idea is the cross-reactivity of different serovars of *P. shigelloides* with various *Shigella* spp. serovars. Thus, *P. shigelloides* O17 shares a somatic antigen with *S. sonnei* (phase I O-antigen); three serovars, O11:H11, O22H3 and O93:H2 cross-react with *S. dysenteriae* serovars 8, 7 and 6 respectively; serovar O23:H1a1c is related to *S. boydii* serovar 13, O54:H2 *to S. boydii* 2, O57:H3 to *S. boydii* 9. In an experiment with rabbits by Sayeed *et al.* (1992), the animals were protected against *S. sonnei* by orally administration *of P. shigelloides* O17. In addition, attempts to develop a vaccine against shigellosis have been done by Taylor *et al.* (1993). These investigators used the O-specific polysaccharide of *P. shigelloides* O17, *S. dysenteriae* type 1 and *S. flexneri* type

2a bound to bacterial toxoids for testing the production of antibodies in humans. Genetic studies (Chida *et al*., 2000) on the region encoding for the O17 antigen in *P. shigelloides* and the form I antigen gene cluster of *S. sonnei* suggest the same origin for both regions. However, it is noteworthy that the physical location for this region in *P. shigelloides* is in the bacterial chromosome whereas a virulence plasmid is the carrier for the cluster in *S. sonnei*.

Furthermore, four additional serogroups of *P. shigelloides* (O5, O14, O15 and O22) have been found identical or related to certain O-antigenic groups of *Aeromonas hydrophila* (O13, O29, O19 and O28) (Shimada & Sakazaki, 1985).

Genotyping

The use of molecular techniques in genotyping bacteria has tremendously increased during the last two decades. Currently, different DNA-based methods (Olive & Bean 1999) have even been introduced as routine analysis in clinical laboratories.

Prior to publication of our studies, there is only one report of molecular typing applied on *P. shigelloides* (Shigematsu *et al*., 2000). However, they only used pulsed-field gel electrophoresis (PFGE) and no information on the serovars of the strains used in that study was provided. Our results (see paper V) showed that the use of DNA-based typing techniques, such as enterobacterial repetitive intergenic consensus (ERIC-PCR), repetitive extragenic palindromic-PCR (REP-PCR), random amplified polymorphic DNA (RAPD) and PFGE, improve the knowledge about the relationship between plesiomonads isolates.

Infections in humans

Since its first isolation in 1947 the number of cases has increased. Although *P. shigelloides* has not been reported in specialised literature as frequently as other enteric pathogens, the increasing number of case reports in recent years is evidence that this pathogen has been overlooked by many laboratories (Clinical Microbiology Proficiency Testing, 2000). This bacterium is generally not considered part of the normal intestinal flora. However, Pitarangsi *et al*. (1982) isolated *P. shigelloides* with similar frequencies from individuals with and without diarrhoeal symptoms in Thailand. Infections caused by this microorganism can be mainly divided in two major groups: one that implicates *P. shigelloides* in gastrointestinal infections and the second as an extra-intestinal pathogen. The former group can be subdivided in three groups depending on the different types of diarrhoea:

A.- Secretory form

This is the most commonly reported form and can last 3-4 weeks. The symptoms are characterised by numerous bowel movements per day.

B.- Invasive shigella-like disease

Rarer than the previous type with abdominal pain and blood in the stool as the main symptoms.

C.- Cholerae-like type

Described in 1986 by Sawle *et al.* (1986) in a patient simultaneously infected with *Aeromonas* spp. and *P. shigelloides*. The symptoms are similar to those caused by *Vibrio cholerae* O1.

There are few reports of outbreaks in the literature: four from USA (Rutala *et al.*, 1982; Miller & Koburger, 1985; Levy *et al.*, 1998), two from Japan (Hori *et al.*, 1966; Tsukamoto *et al.*, 1978), one in Cuba (Bravo *et al.*, 2000) and one in The Netherlands (Medema & Schets, 1993). However, a series of case reports have been communicated from other countries in tropical and subtropical areas such as Bangladesh (Albert *et al.*, 1999), India (Chatterjee & Neogy, 1972; Saraswathi *et al.*, 1983), Malaysia (Issa *et al.*, 1999) Thailand (Echeverria *et al.*, 1985; Taylor *et al.*, 1985), Zaire (van Damme & Vandepitte, 1980), Australia (Cooper & Brown, 1968; Buckley *et al.*, 1998), Mali (Vandepitte *et al.*, 1980), Kenya (Jandl & Linke, 1976), Mexico (Jiang *et al.*, 1991), Nigeria (Obi *et al.*, 1995; Obi *et al.*, 1997), Madagascar (Zeller *et al.*, 1978) and Peru (Olsvik *et al.*, 1990). In addition, several temperate countries as Canada (Kain & Kelly, 1989), Spain (Reina & Serra, 1993; Ruiz *et al.*, 1995), Czechoslovakia (Karolcek *et al.*, 1982), Finland (Rautelin *et al.*, 1995), Sweden (Svenungsson *et al.*, 2000) and South Africa (Obi & Bessong, 2002) have reported gastrointestinal infections *by P. shigelloides*. Although there is evidence that some cases are produced by autochthonous plesiomonads most of the patients in these countries presented a history of traveller's diarrhoea to the so-called "high-risk zones" (DuPont & Ericsson, 1993).

There are several reports on the role of *P. shigelloides* in extra-intestinal infections. The majority of these cases are septicaemia in immunocompromised patients, many of them with a fatal outcome. Neonates are the most affected age group and according to Claesson *et al.* (1984), *P. shigelloides* can cause unusual clinical pictures once the microorganism invades the body. Other extra-intestinal infections caused by *P. shigelloides* are pyosalpinx (Roth *et al.*, 2002), cellulitis (Jönsson *et al.*, 1998), migratory polyarthritis (Gupta, 1995), ocular infection (Butt *et al.*, 1997), acute cholecystitis (Claesson *et al.*, 1984).

Infections in animals

If water is considered the main reservoir of *P. shigelloides*, aquatic organisms, water-dwelling reptiles and birds may serve as a second reserve for this bacterium. Plesiomonads have been isolated from various animal species (Bauwens *et al*., 1983; Bardon, 1999a; Bardon, 1999b). Both, poikilotherms and homeotherms can harbour this organism. However, its pathogenic role in veterinary medicine is even more controversial than in human medicine because of the lack of clinical reports. Most of the evidences suggesting that *P. shigelloides* is a human pathogen are based on the isolation of this bacterium from patients with diarrhoea. Similar to the spectrum of diseases caused by this pathogen in humans, symptoms in animals varies from catarrhal and haemorrhagic enteritis to sepsis (Bardon, 1999a).

In 1954, *P. shigelloides* was isolated from the spleen of a chimpanzee in a post mortem examination (Schmid *et al*., 1954), but no pathological data was presented in relation with the death of the animal and the presence of this bacterium. These researchers found *Shigella flexneri* in the kidney of the same animal. Later, Eddy and Carpenter (1964) isolated this microorganism from different animal species such as dog, red howling monkey, puppy, cat and xenopus toad. Once more, information about the cause of the decease of the animal and its possible relation with isolation of *P. shigelloides* was missed. Davis II *et al*. (1978) described a case in which a man working at a zoo was supposedly infected by *P. shigelloides* (called *Aeromonas* (Pl) *shigelloides* in this article) from a boa constrictor. The investigators associated the death of the snake with the presence of *Aeromonas* spp. However, although the cultures from the animal lesions (pre mortem and post mortem) yielded *Aeromonas* spp., the lack of biochemical test to identify the specific species does not give a total confidence to affirm that this is a new zoonosis.

A survey of *P. shigelloides* was carried out in Japan during the period 1974-1978 in order to determine the distribution of this bacterium in, among other sources, cattle, swine, poultry, dogs, cats and fresh water fish (Arai *et al*., 1980). The microorganism was isolated from 37 dogs (3.8% of 967) and 40 cats (10.3% of 389). Most of these animals were asymptomatic. Further, they could not isolate plesiomonads from healthy cattle, swine and poultry. Interestingly, because many of the strains isolated from dogs and cats were of the same serovars as those identified from patients with diarrhoeic symptoms they suggested that these two species might play a role in human infections. Our investigations (paper III and V) agree with those from Arai et al.

In a study carried out in Belgium (Bauwens *et al*., 1983) 254 faecal samples were collected from different animal species in a zoo and examined for the presence of *P. shigelloides* and *Edwardsiella* spp. According to their results, *P. shigelloides* was isolated from a few animals with diarrhoea and additionally from a sample from a lung of an otter. However, plesiomonads were frequently isolated in the Antwerp Zoo, being birds (mainly from pinnipeds and gulls) and bears the animals where this bacterium was isolated in great numbers.

Bardon (1999a) carried out an evaluation of the pathogenicity of *P. shigelloides* in animals. He found plesiomonads in 55 cases, which represented 1.21% of the 4.552 samples examined. However, 60% of the strains were isolated during investigations of the death or the disease of the animal. Interestingly, cats ranked first among mammals from which *P. shigelloides* were isolated. This finding agrees with the results presented by Arai *et al*. The work of Bardon showed that fishes -mainly aquarium fishes and trout- presented the highest percent of cases in which this pathogen were isolated.

A very interesting paper was published in 1998 by Sparkes *et al*. (1998). They studied the effect of fructo-oligosaccharides (FOS) as dietary supplementation on the faecal flora of healthy cats, and found *P. shigelloides* among the most frequently isolated aerobic bacteria. Surprisingly, they did not discuss the fact that, *P. shigelloides* (like lactobacilli) increased in prevalence and numbers after supplementation with FOS but this was not the case for *E. coli*. Because plesiomonads are thought to be a potential pathogen in cats, the increase of this bacterium due to the specific diet contradicts the conclusions they drew in this paper.

Only two outbreaks occurring in fish are described in the literature (Vladik & Vitovec 1974; Cruz *et al*., 1986). Both papers reported high mortality in trouts. In the earliest one, isolates not only showed *P. shigelloides*, but also *Flavobacterium* sp. and *Aeromonas hydrophila*. Yet, *P. shigelloides* was the only bacterial species isolated by Cruz and co-workers in 1986.

In the Nordic countries, the isolation of *P. shigelloides* from either diarrhoeic symptomatic or asymptomatic animals is rather limited. Only one case (a cat with diarrhoea) in Norway (Sjöberg, 1994); twelve animals in Finland (Niskanen & Salmela, 2000); one dog, three cats and five fishes in Sweden (unpublished results). To my knowledge, there is not data available from Denmark and Iceland.

Pathogenesis and virulence factors

The route of entry into the human and animal gastrointestinal tract seems to be the ingestion of contaminated food or water (Fig. 3). Symptoms associated with gastroenteritis caused by *P. shigelloides* are diarrhoea, abdominal pain, nausea, chills, headache, fever and vomiting. Although there are reports of bloody stools (Olsvik *et al*., 1990; Ahmad *et al*., 1998), most of the stools from patients with diarrhoea are described as watery. Incubation time varies from 24-50 hours and symptoms generally last for 1-9 days, although a more invasive *Shigella*-like type can last from 2 weeks to 3 months (Clark & Janda 1991).

Various virulence factors have been studied to determine the pathogenic mechanisms of infections caused by *P. shigelloides*. These have not yet been fully elucidated. Contradictory results from different studies and the lack of an animal model hinder research progress. However, Vitovec *et al*. (2001), studying co-infection with *P. shigelloides*, *Aeromonas* spp. and *Cryptosporidium parvum*,

suggested that the neonatal BALB/c mouse might be a valid experimental model for the study of gastrointestinal disease caused by bacterial and protozoan organisms.
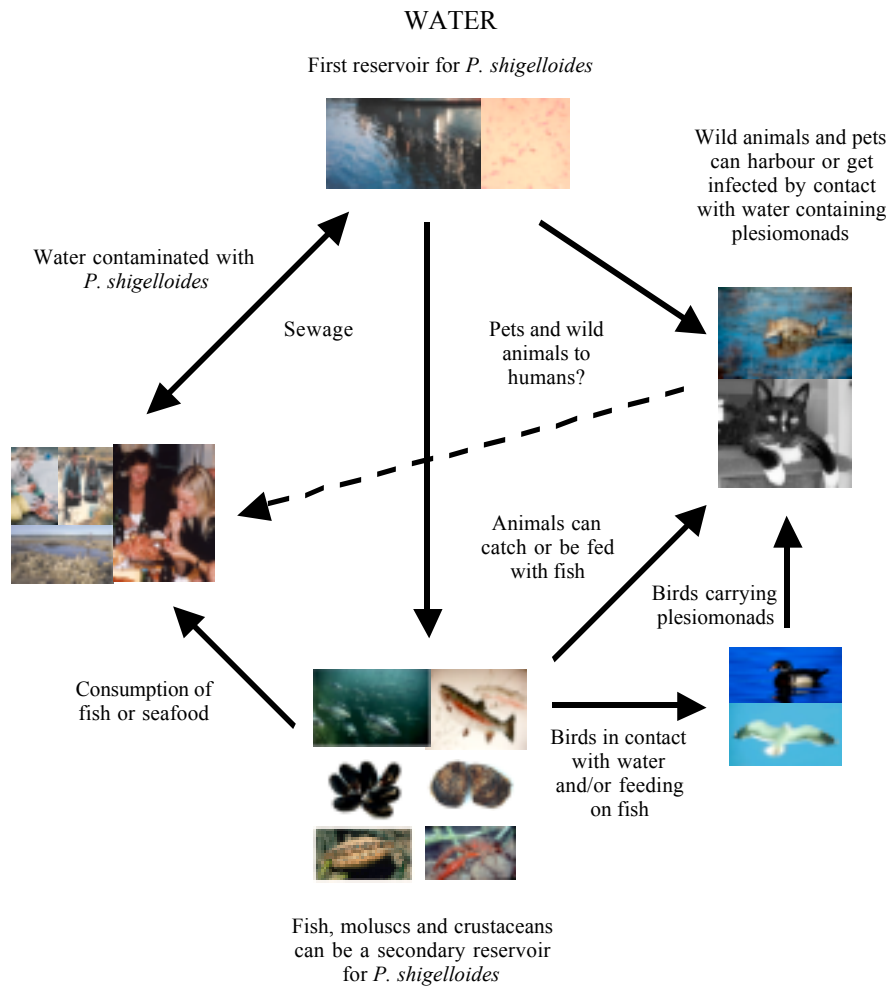

Fig. 3. *P. shigelloides* infection pathways.

Adhesion

Schubert & Holz-Bremer (1998/1999) working with isolates from human and environmental sources found that strains from clinical material showed higher adhesion level than those from the aquatic environment. It is noteworthy that, in a

study carried out at our laboratory, Ekman (2003) detected a completely different behaviour among the tested strains (Fig. 4). Then, the bacteria from aquatic environment generally presented higher adhesion ability compared with those from Schubert & Holz-Bremer. Variations in experimental conditions as well as geographical and serological differences may explain these contradictory results.
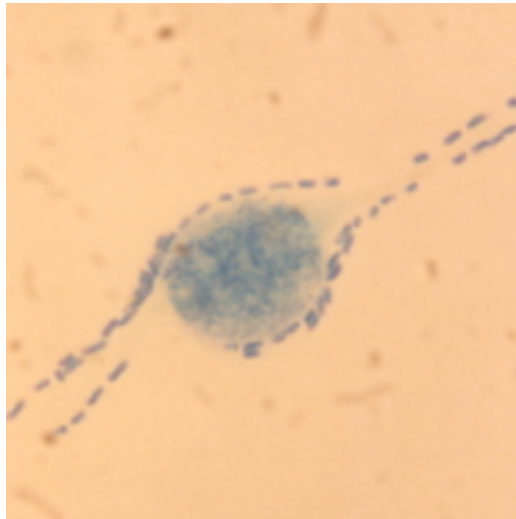

Fig. 4. Adhesion of *P. shigelloides* to an INT-407 cell. (Methylene blue stain. Light microscopy X 1250). Photo: Karel Krovacek.

Invasiveness

The ability of *P. shigelloides* to invade cells has also been studied with contradictory results. Some investigators obtained positive results (Binns *et al*., 1984; Olsvik *et al*., 1985) while some others did not find any invasive potential for this microorganism (Sanyal *et al*., 1980; Herrington *et al*., 1987). It was not until 2001 that Theodoropoulos *et al*. (2001) demonstrated, by means of transmission electron microscope and using human epithelial cells (Caco-2), that plesiomonads are able to enter gastrointestinal cells. They also suggested that differences in invasion phenotypes might be due to different pathogenic phenotypes.

Enterotoxin

The production of enterotoxin is another controversial issue in the study *of P. shigelloides* potential virulence factors. Once more, investigators have obtained variable results. Manorama *et al*. (1983) characterised and partially purified two toxins, heat-stable (ST) and heat-labile (LT) enterotoxins. However, researchers have demonstrated the production of enterotoxin in vivo using, mainly, two models: rabbit ileal loop model (Fig. 5) (Sanyal *et al*., 1975; Huq & Islam, 1983; Manorama *et al*., 1983; Matthews *et al*., 1988; Dumontet *et al*., 1998) and

suckling mouse assay model (Sanyal *et al.*, 1980; Huq & Islam, 1983; Manorama *et al.*, 1983; Saraswathi *et al.*, 1983; Abbott *et al.*, 1991). Unlike the former authors, Herrington *et al.* (1987) did not find any production of enterotoxin when they tested their isolates by GM1-enzyme-linked immunoabsorbent assay (ELISA).
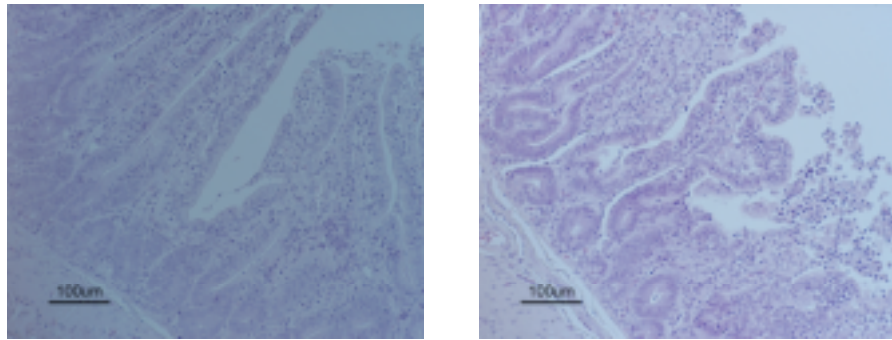

Fig. 5. Histopathological examination of the rabbit intestine inoculated by whole bacterial cells of *P. shigelloides* isolated from fresh water (left) or from human case of diarrhoea (right). Notice the damage intestinal mucosa with markedly shortened or irregular shaped villi and the extensive erosion because of necrosis and exfoliation of the epithelium lining the apical half of the villi (right). Photo: Ricardo Feinstein.

The attempt to find sequence homology with well-characterised enterotoxin genes of other bacteria species has failed (Matthews *et al.*, 1988). They therefore suggested that this *P. shigelloides* toxin is a new class of heat-stable toxin.

Cytolysins

Several studies have shown the cytotoxic effect of *P. shigelloides* on various cell lines. Thus, Olsvik *et al.* (1990), Abbott *et al.* (1991), Dumontet *et al.* (1998) and Ekman (2003) could demonstrate cytotoxic/cytotonic effects on Vero cells (Fig. 6). Y1 cells have been used by Abbott *et al.* (1991), Sanyal *et al.* (1980) and
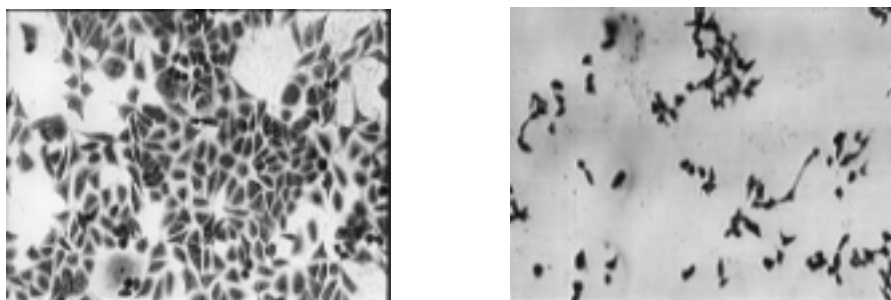

Fig. 6. Vero cells (left) showing cytotoxic effect (right) after inoculation with filtrate of *P. shigelloides*. Photo: Karel Krovacek.

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Gurwith & Williams (1977). In addition, studies using Hep-2 (Abbott *et al.*, 1991) and INT-407 (Ekman 2003) have shown that these cell lines can also be strongly affected by the cytotoxic effect of some strains of this microorganism (Fig. 7). Examining 29 strains of *P. shigelloides* in CHO cell cultures, Gardner *et al.* (1987) detected the presence of a cholera-like toxin. This substance caused
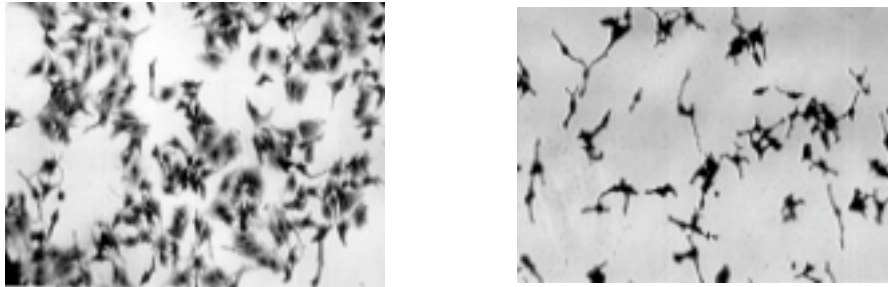

Fig. 7. INT-407 cells (left) showing cytotonic effect (right) after inoculation with filtrate of *P. shigelloides*. Photo: Karel Krovacek.

morphological changes in CHO cells resembling that produced by cholera toxin and *E. coli* heat-labile toxin. It is noteworthy that these investigators used iron-depleted medium for growing plesiomonads. No elongation of CHO cells was observed when they grew *P. shigelloides* on other media.

Haemolysin

The detection of haemolysin in bovine, horse, sheep and rabbit blood agars has been questionable. Although investigators such as Krovacek *et al.* (2000), Aldova *et al.* (1966), Zajc-Satler *et al.* (1972) have detected haemolysis, most of the studies revealed negative results (Hassan *et al.*, 1994; Pitarangsi *et al.*, 1982). However, the use of other methods for detection of haemolytic activity showed that, in fact, *P. shigelloides* can produce haemolysin. Santos *et al.* (1999) detected extracellular haemolytic activity using iron-depleted tryptic soy broth (TSB). Their results and those obtained by Baratela *et al.* (2001) suggest that the production of protein with haemolytic activity might be regulated by iron limitation. The latter workers also proposed that media composition, oxygen tension and temperature influence haemolysin expression. Janda & Abbott (1993) used two different methods to screen the ability of *P. shigelloides* to produce haemolysis. In the first, an agar overlay containing human type A erythrocytes was used. In the second, bacteria suspension and 2% of human erythrocytes were incubated in a 96-well microtiter plate and read at hourly intervals. More than 90% of the strains tested produced a product with haemolytic activity and this protein was active against a variety of erythrocytes.

Elastin

Elastolytic enzymes have the ability to degrade connective issue and therefore they might contribute to the pathogenicity of microorganisms (Rust *et al.*, 1994). Santos *et al.* (1999) found elastolytic activity with cell suspensions. This activity

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was enhanced when the bacteria were previously cultured in iron-depleted TSB. Ciznar and co-workers found plesiomonads strains producing elastolytic enzymes using a quantitative elastin-Congo red test (unpublished data).

Plasmids

In various studies, researchers have investigated the presence of plasmids and their significance in the process of infection by *P. shigelloides*. While some investigators have detected only a very large plasmid in strains from clinical sources (Herrington *et al.*, 1987), others found small plasmids ranging from 2 to 8 MDa (Abbott *et al.*, 1991; Marshall *et al.*, 1996; Bravo *et al.*, 1998). The first evidence that these genetic elements might be involved in *P. shigelloides* pathogenic mechanisms was presented by Herrington *et al.* (1987). They observed that when a gnobiotic piglet was infected with a plasmid-cured strain the animal did not get sick. It was suggested that high molecular plasmids might be involved in the process of invasion.

Another property that has been shown to be plasmid-mediated is resistance to antibiotics. However, to date, only one research group has succeeded in demonstrating this. Marshal *et al.* (1996) found two plasmids in *P. shigelloides* isolated from blue crab associated with resistance to streptomycin. Furthermore, Avison *et al.* (2001), determined the sequence of a cryptic plasmid in a strain of *P. shigelloides* that might encode a bacteriocin.

Other proteins that can cause illness:

Tetrodotoxin

Tetrodotoxin (TTX) is a marine neurotoxin. Shellfish can contain this TTX and it has been reported an outbreak in Taiwan due to the consumption of molluscs containing TTX (Wei *et al.*, 1994). It has been shown that this toxin can be produced by different bacteria species, mainly *Vibrio* spp. (Narita *et al.*, 1987; Simidu *et al.*, 1987; Hwang *et al.*, 1989). Cheng *et al.* (1995), studying the microflora of the shellfish *Niotha clathrata*, found *P. shigelloides* among other microorganisms. These workers tested the bacterial isolates for production of TTX, revealing *P. shigelloides* as one of the TTX producers.

Histamine

Scombroid poisoning is a very common intoxication associated with ingestion of seafood or fish containing high concentrations of histamine (Lopez-Sabater *et al.*, 1996; Lipp & Rose, 1997). Various bacteria species have been associated with the production of this protein (Yoshinaga & Frank, 1982; Middlebrooks *et al.*, 1988; Lopez-Sabater *et al.*, 1996) The latter researchers suggested an important role for *P. shigelloides* because of its association with the aquatic environment. In a recent investigation we found that 13.3% of the investigated plesiomonads were able to produce histamine (unpublished data).

Antibiotic patterns

Like other members of the family *Enterobacteriaceae*, most of the *P. shigelloides* strains are resistant to a broad spectrum of penicillins, including ampicillin, piperacillin, ticarcillin, mezciocillin, carbenicillin, azlocillin and others (Stock & Wiedemann, 2001b; Avison *et al*., 2000). There is a strong co-relation between the inoculum and the medium used for testing β-lactam susceptibility *in P. shigelloides* (Stock & Wiedemann, 2001a), a fact well known in members of the family *Enterobacteriaceae*. In addition, this resistance to penicillins has not arisen from the selective pressure of β-lactam products. In a study by Avison *et al*. (2000) some of the strains used in this investigation, and that displayed resistance to penicillins, were isolated in the mid-1960s, before the drugs carbenicillin, piperacillin and oxacillin were developed. This phenomenon indicates that a pool of β-lactamases is present in *P. shigelloides* in the environment. There is strong evidence that suggests a new species-associated expression mechanism (Avison *et al*., 2000; Stock and Wiedmann, 2001a). The mechanisms of resistance to β-lactams in plesiomonads have not been elucidated yet.

Results on the susceptibility to aminoglycosides are variable. In a study by Kain & Kelly (1989), *P. shigelloides* showed resistance to amikacin, gentamicin and tobramycin. Clark *et al*. (1990) only detected a few strains resistant to gentamicin and tobramycin and none to amikacin. Marshall *et al*. (1996) found that strains isolated from blue crab were susceptible to gentamicin. Moreover, a recent work (Stock & Wiedemann, 2001b) revealed that *P. shigelloides* strains can be resistant to other aminoglycosides as well. Streptomycin and lividomycin A showed an intermediate activity and some strains isolated from humans and animals can display resistance to apramycin. The mechanism for aminoglycoside resistance in *P. shigelloides* is still unknown, although the most common way to escape the action of these agents is by enzymatic inactivation by amino-glycoside-modifying enzymes (Shaw *et al*., 1993).

Plesiomonads are generally sensitive to second- and third-generation cephalosporins (except cefoperazone, ceftazidime and cefepime), carbapenems, aztreonam, trimethoprim + sulfamethoxazole, fosfomycin nitrofurantoin, nalidixic acid, co-trimoxazole, chloramphenicol and quinolones (Stock & Wiedemann, 2001b). However, Wong *et al*. (2000) detected strains resistant to co-trimoxazole and chloramphenicol.

Results of various studies show that *P. shigelloides* is naturally resistant to a diversity of β-lactams. For this reason, it would be advisable to avoid these antibiotics for treating infections caused by this microorganism; quinolones and potentiated sulphonamides might be used instead. The same recommendations should be taken for veterinary medicine. Data from cats with recurrent diarrhoea and treated with penicillins suggest that plesiomonads remain in the intestinal tract or inside the cells until they are triggered again (González-Rey *et al*., unpublished data).

Aims

The aims of the present work were:

To investigate the occurrence of *P. shigelloides* in aquatic environments in Sweden.

To develop a PCR assay for detection and identification of *P. shigelloides* from aquatic environments, animals and humans.

To evaluate the antibiotic resistant patterns in *P. shigelloides* isolated from various sources in selected geographical regions.

To evaluate several DNA-based techniques to differentiate strains of the same serovar.

Comments on materials and methods

A summary of the materials and methods used for the present study are listed below. For a more detailed description, see Papers I-V.

Bacterial sampling (I-V)

Humans

The strains used in these studies were isolated from patients suffering gastrointestinal infections. Stools were sent to the local laboratories for detection, identification and characterisation. The studies included patients from the following countries: Finland, Cuba and Czech Republic.

Animals

The animal samples were taken in Cuba and Finland. The bacteria were isolated from faeces and internal organs both from living animals and those examined post-mortem. Some animals did not show any clinical sings. The isolates were previously identified at the National Veterinary and Food Research Institute (EELA), Finland and at the Institute of Tropical Medicine "Pedro Kouri", Cuba.

Environment

Environmental strains were isolated from fresh and salt water from Sweden, Slovak Republic and Italy. Samples were taken from approximately 20-30 cm below the water surface in sterile glass bottles. The samples were transported to the respective laboratories for bacteriological analysis. Water temperature and pH were measured at the sampling sites.

Bacterial culture and identification (I-V)

All samples were streaked onto blood agar containing horse or bovine erythrocytes. The environmental samples were previously enriched in peptone water as described in paper I. Bacteria isolates from other countries were confirmed at our laboratory in order to avoid possible contamination with other bacteria species. Isolates that were Gram negative, oxidase, indol, maltose and glucose positive were further characterised by biochemical typing using the API 20E system. The strains were further tested by means of the specific PCR for detection of *P. shigelloides* developed in our laboratory (see paper IV).

Antibiotic susceptibility (III)

The disk diffusion method was used in this work for determining antibiotic susceptibility. This is a qualitative technique that allows classification of the microorganism as either susceptible, intermediate or resistant. In the present study the resistance or susceptibility of 73 strains of *P. shigelloides* to eighteen antibiotics (amikacin, gentamicin, netilmicin, tobramicin, amoxicillin +

clavulanic acid, ampicillin, aztreonam, imipenem, mezlocillin, cephalothin, cefotaxime, ceftazidime, ciprofloxacin, ofloxacin, doxycicline, tetracycline, chloramphenicol, trimethoprim + sulfamethoxazole) and the vibriostatic agent O/129 were examined. The test used in this investigation was performed according to the National Committee for Clinical Laboratory Standards (NCCLS).

Serotyping (I-V)

Relatedness between the different bacterial strains is important for establishing hypothesis based on epidemiological studies. Serotyping has been the most commonly used method of election for typing *P. shigelloides*. The serological typing was performed according to the international antigenic (Shimada *et al*., 1994; Aldova & Shimada 2000) and by the provisionally called "Schubert" scheme as described by Aldova and Schubert (1996) using specific *Plesiomonas* antisera. O- and H-sera were prepared at the National Institute of Sera and Vaccines, Prague, Czech Republic. Briefly, O-antisera were prepared by immunising rabbits with 18-hour broth cultures boiled at 100 ^{o}C for 2 hours. Rabbits were injected at 4-days intervals with increasing volumes of the antigen suspension. These animals were bled 7 days after the last immunisation. For H-antisera, cultures in which motility was promoted by serial passage through semisolid medium were used. To remove the O antibody, we performed absorption with the homologous cultures heated at 100 oC for 2 hours. The immunisation and bleeding schedule was the same as that for preparation of O-antisera. All positive slide agglutinations were confirmed in tubes.

DNA preparation (II, IV, V)

Because the purity of the DNA is very important for obtaining reliable results when PCR-based methods are used, the choice of an adequate technique for isolation and purification of the bacterial DNA is crucial. Genomic DNA was extracted from bacterial cells using a method previously described by Wilson (1994). Briefly, two ml of bacterial cells grown in brain heart infusion broth was centrifuged. The pellet was resuspended in TE buffer containing sodium dodecyl sulfate and proteinase K. Next, NaCl and CTAB were added. The CTAB-protein/polysaccharide complex formed during the process is removed by phenol-chloroform extraction. Concentration and purity of the DNA was measured by the Gene Quant system. Working dilutions (10 ng x l^{-1}) were prepared for the experiments.

Determination of the 23S rRNA target sequence, design of the species-specific primers and PCR assay (IV)

The use of the BLAST program (Altschul *et al*., 1997) was fundamental for the searching of regions with enough number of gaps that might help in the design of species-specific primers. A commercial primer analysis program called OLIGO 5.0 was used to assist in the selection of the two primers. The selected primers

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amplified the region between the nucleotide C-906 and G-1189 of the 23S rRNA gene.

The key for the PCR was the high temperature used for annealing (68 ^{o}C). This temperature prevents amplification of non-plesiomonad genetic material.

The PCR products were sequenced and aligned with the CLUSTAL W1.7 program (Thompson *et al.*, 1994).

Random amplified polymorphic DNA (RAPD) (II, V)

RAPD is based on the used of short random-sequence primers that hybridise at low annealing temperature with the DNA present in the PCR reaction (*Williams et al.*, 1990; Welsh & McClelland, 1990). To avoid reproducibility problems, we maximized the standardisation of this technique in our studies by using a commercial kit designed for RAPD. This kit contains beads with all the necessary reagents, except the primer and the target DNA, thus minimizing the risk of pipetting errors.

The annealing temperature was low (36 ^{o}C) to allow primers to bind to the DNA with sufficient affinity to amplify regions of the bacterial genome.

Pulsed-field gel electrophoresis (PFGE) (II, V)

PFGE is considered the "gold standard" of the DNA-based typing methods (Maslow *et al.*, 1993). A restriction enzyme is used to digest the bacterial DNA. The bacterial cells are first embedded in agarose. This procedure avoids the random cutting of the DNA that might occur during the mechanical process of isolation and purification. Once the DNA has been digested, the plugs are inserted in an agarose gel and an electrophoresis, where the electrical impulses change at different intervals and from different angles, is performed. The gel is afterwards stained with ethidium bromide and DNA bands are visualised by means of UV light.

One of the advantages with this kind of electrophoresis is that very large-size fragments of DNA can be efficiently separated in an agarose gel. Reproducibility of the results is another valuable benefit of this method, allowing creation of databases for further epidemiological studies.

Results and discussion

Isolation, biochemical and serological characteristics of *P. shigelloides* isolated from fresh water (I, III, IV)

Freshwater samples from central part of Sweden were taken in order to detect the occurrence of *P. shigelloides*. The five isolates produced β-haemolysin when they were grown on blood agar containing bovine erythrocytes. The haemolytic activity was negative when horse erythrocytes were substituted. The isolates were biochemically and serologically characterised. The biochemical profiles were identical for all the strains indicating that the API 20E system is reliable for identification of this microorganism. Furthermore, serotyping revealed that our isolates belonged to serovars O18:H3, O22:H3, O23:H1a1c, H1a1b, O26:non-agglutinating H, O58:H22, O60:H2 and O66:H3. Although Aldova (1994) could not find in Europe and Asia the serovars O23, O58 and O60 among strains isolated from different sources, Bardon (1999) found O23 and O60 in fishes in Central Europe in a study on serovars in animals in the Czech Republic. This is an interesting finding, as these specific serovars have not been found in isolates from non-aquatic animals.

Plesiomonas sp. has previously been isolated from patients and animals in Nordic countries (Sjöberg, 1994; Rautelin *et al*., 1995; Jönsson *et al*., 1998; Niskanen & Salmela, 2000), but the presence of this microorganism in Nordic fresh water has never been studied. This is the first report on the isolation of this enteropathogen from fresh water in Northern Europe. Andersson & Stenström (1987) reported that waterborne outbreaks are very uncommon in Sweden. The authors stated that the number of reported cases is an underestimation of the true number of outbreaks, and that many of the outbreaks reported in this country are of unidentified aetiology. This finding agrees with the 1996 annual report of the Swedish Institute for Infectious Disease Control that shows a large number of cases of food- and water-borne infections could not be ascribed to any particular agent. Therefore, at least a part of these unknown food- and water-borne cases of human and animal enteritis could be due to emerging pathogens, including *P. shigelloides*.

P. shigelloides in the Swedish Arctic Area (II)

The results of the water samples taken from six lakes above the Polar Circle showed the presence of *P. shigelloides* in one of the investigated lakes. As in the previous study, the isolates displayed haemolytic activity when they grew on blood agar plates containing bovine erythrocytes. All the isolates presented the same biochemical profile and the serotyping showed just one serovar, namely O19. Interestingly, all the isolates were non-agglutinating for the H-antigen. Two of the strains were non-agglutinating for neither the O-antigen nor the H-antigen. Because the O19 strains could be different for the H antigen, and there were two non-agglutinating strains, we decided to perform RAPD and PFGE in order to examine the genetic relationship between the different strains. This is the first time that RAPD has been used for typing *P. shigelloides*. PFGE has only been used

once before this work (Shigematsu *et al*., 2000), but there was no information on the serovars to which the isolates belonged. Our results showed that both techniques could discriminate between the strains, indicating a genetic variability among the plesiomonads isolated from the same lake. In addition, serotyping, RAPD and PFGE showed that the serovar O19:NA, or what we called genotype 1, was predominant among the strains we isolated from Lake Vattasjärvi.

This finding is important from an ecological point of view as well as epidemiological perspective. Not only does *P. shigelloides* occur normally in Swedish fresh waters but it can also be found in polar environments. Moreover, it shows the ability of this bacterium to establish in habitats where conditions are very unfavourable for long periods. Thus, an important question arises: how plesiomonads can resist such adverse environmental conditions? *Because P. shigelloides* does not produce spores, other mechanism(s) must be involved in the way(s) this microorganism survives. There are two possible explanations. The viable but non-culturable state suggests a mechanism by which microorganisms switch over to the VBNC form when the conditions are hostile. Several researchers have showed that cold- and starvation stress may induce microorganisms to change into this non-culturable state. There is evidence suggesting that formation of VBNC cells may be one of the strategies adopted by bacteria for survival the extreme Arctic environmental conditions. The other mechanism implies the production of a disaccharide called trehalose. It has been shown that trehalose can protect cells from destruction when they are under stressful conditions. Moreover, *P. shigelloides* is known to produce this type of non-reducing disaccharide.

How its presence can affect the "normal" microflora community of that extreme climate, and the role which plesiomonads play in relation with animals from the polar aquatic environment or terrestrial fauna that contact water in *which P. shigelloides* lives, are questions, among others, to be studied in the future. It is clear that our view of *P. shigelloides* as a tropical/subtropical or "Asian pathogen" bacterium must be modified.

Serotypes and antibiotic susceptibility patterns of *P. shigelloides* isolated from different geographical areas (III)

The results of the serotyping of the strains isolated from human, animal and environmental sources suggest a high phenotypic and therefore genomic variability. These results agree with previous studies where *Plesiomonas* sp. showed a broad diversity in serovars. Furthermore, most of the serovars presented a random geographical distribution, which is in agreement with previous studies. However, 31.3% (5/16) of the strains isolated from animals in Finland displayed the association O66:H3. An interesting finding was that neither cats nor dogs showed this serovar. Antigen H3 was the most represented (52.4% of the total animal strains; 68.8% of the Finnish isolates). The sum of the H3 + H2 antigens represented 76.2% of the total antigens. In humans, serovar O40 was only found in Finnish patients as well as the H6 antigen. The combination O40:H6 was present in 25% of the Finnish patients and represented 75% of the H6 antigens. The antigen H11 was only displayed in strains isolated both from human and

animal sources in Cuba. Strains isolated from environmental sources did not show any particular association between the serovar and the geographical region. Furthermore, some serovars were present in both human and animals, predominantly in cats, in the same country. These results suggest that some serovars can actually be predominant in some countries. On the other hand, those serovars may have been "selected" by infecting organisms and what we see is only a reflection of the ability of these particular serovars to colonise animals and/or humans. Moreover, the finding that the same serovar was found in animals and humans from the same country strengthens the hypothesis, previously suggested by other researchers, that *P. shigelloides* is involved in zoonotic cases.

The strains of this work were also tested for their susceptibility to eighteen anti-microbial agents and the vibriostatic agent O/129. The importance of knowing which antibiotics can kill this pathogen is fundamental for human and veterinary medicine, as well as for environmental sciences. In general, our results on the resistance to ampicillin and mezlocillin are in accordance with those obtained by other authors (Stock & Wiedemann, 2001b). However, discrepancies were found for aztreonam, imipenen or amoxicillin + clavulanic acid. This might be due to the number of bacteria used as inocula.

Our results showed a tendency to present resistance to tetracyclines by strains isolated from human sources. These strains were isolated from Finnish patients and the fact that these isolates were serologically different to those isolated from animals in Finland might suggest that the bacteria were acquired elsewhere geographically. Moreover, the isolates from Finnish animals were susceptible to tetracyclines, as were all the isolates from Cuban patients. However, De Paola *et al*. (1993) showed that *P. shigelloides* contains genes that code for tetracycline resistance. It may be that these genes are not expressed under the experimental conditions within our study

One strain isolated from a dog in Cuba was resistant to various antibiotics. This example should alert us to the importance of antibiotic control in veterinary medicine.

Specific detection of *P. shigelloides* by PCR based on 23S rRNA gene (IV)

Specific detection of bacteria using the 23S rRNA gene has been previously studied. Analysis of this region in various microorganisms shows more variation than the 16S rRNA. However, the availability of new data for 23S rRNA sequences has increased during recent years. The objective of this study was to develop a PCR method that could detect *P. shigelloides* specifically. For that purpose, we used two primers targeting part of the 5' half of the 23S rRNA gene. The results of the PCR assay and electrophoresis showed a fragment of 284 bp in length corresponding with the expected size. Amplification products were obtained from all the *P. shigelloides* strains used in this investigation, from all geographical origins, different sources of isolation, and various serovars. The DNA fragments were further sequenced in both senses and the sequences obtained were

in accordance with the appropriate region of the sequence previously determined for the 23S rRNA gene.

Various additional bacterial species, closely related genetically to *P. shigelloides*, were tested in this work. The results showed no amplification of these microorganisms; therefore, we concluded that this method was reliable for distinguishing between *P. shigelloides* and other bacteria species.

Evaluation of DNA-based techniques for *P. shigelloides* genotyping (V)

Several studies have been done to elucidate clonal relations between phenotypically identical strains. Biochemical tests, antibiotic resistance profiles, serotyping, phage typing and multilocus enzyme electrophoresis are the most common tools used for phenotypic characterisation. However, none of these techniques are reliable for discriminating between isolates within species or they are limited to just a few reference laboratories. Techniques based on DNA can discriminate at a deeper level. This study describes how molecular techniques can discriminate between strains that are of the same serovar. DNA techniques minimize the problems with typeability and reproducibility. RAPD, REP-PCR, ERIC-PCR and PFGE have been used for a large number of studies on molecular epidemiology (Olive & Bean, 1999).

Our results showed that some strains isolated from the same geographical area and isolated from human and animal sources, displayed the same pattern profile, which suggests a clonal relationship.

Although there are indications of a high diversity in the plesiomonads population, we could find three cases in which patterns from isolates from animals and from humans were equal or highly similar. This finding is relevant because it shows that the same genotype can infect humans and animals.

It is known that having contact with water and/or food contaminated with this bacterium can infect humans and animals but the role that pets or wild animals play in the spread of this microorganism is still unclear. Whether the isolates from animals and humans that presented the same genotype were infected by direct contact with the same water and/or contaminated food or the animals carried the bacteria directly to humans is difficult to determine. However, this study confirms that isolates with the same genetic profile can infect both animals and humans. Moreover, strains within the same serovars can be differentiated at genomic level so we can hypothesise that the same clone was not responsible for the infection in both animals and in humans.

General discussion

For many years, *P. shigelloides* has been considered a bacterium of tropical and subtropical countries. Results presented in this thesis indicate *that P. shigelloides* has in fact, a global distribution. The biological and ecological questions raised by our discovery of *P. shigelloides* in a lake north of the Polar Circle provide interesting challenge for future studies.

The potential risk for humans and animals in contact with water containing this emerging pathogen must be further evaluated. Because *P. shigelloides* can be overlooked by clinical laboratories or can be part of a co-infection, techniques for plesiomonads detection based on DNA can help to monitor better the presence of this organism in clinical specimens. For that reason, the development of a PCR for specific detection was one of our goals that was accomplished.

Antibiotic susceptibility testing has mainly used strains from human and environmental sources. Because there are studies showing that, in fact, *P. shigelloides* is also an animal pathogen, we used strains isolated from various animals for testing their susceptibility to a variety of antibiotics.

Serotyping and genotyping of microorganisms is an important issue for epidemiological studies. Until now, there were only studies based on differences or similarities at serovar level. Relationships could not be established in all cases. For example, the absence of agglutinating reactions for some strains excluded this possibility. To avoid this problem, molecular techniques were used for the first time in order to find relationships between strains of the same serovar. It is significant that the use of these techniques allowed us to distinguish between strains presenting the same serovar. Moreover, strains of the same serovar but from different sources displayed the same genotype, suggesting that *P. shigelloides* might be involved in zoonotic cases. This work presented the first evidence of this phenomenon at molecular level.

In conclusion, this thesis research has contributed significantly to our knowledge of *P. shigelloides* by providing new information on its distribution, its specific detection by PCR, intra-species relationship and its possible relation with zoonotic cases.

Future investigations

Investigations into the role that *P. shigelloides* play in gastrointestinal infections in animals, and particularly in cats, have already commenced. Our preliminary results show that *P. shigelloides* can be found in cats with recurrent diarrhoea. Because these cats were treated with penicillin, it might be speculated, considering our preceding investigations on antibiotic susceptibility, that plesiomonads survive the treatment either by remaining in the intestinal environment or by being sequestered within the gut cells.

The role that different proteins play in the mechanisms that *P. shigelloides* use to cause illness or to escape the immune system is also poorly understood. Variation of the cultural conditions lead to the expression of different proteins. The identification and characterisation of these proteins can help to reveal how plesiomonads behave when environmental factors change. For that purpose, we are going to use 2D-electrophoresis in future studies in order to obtain information about which proteins are expressed when the bacteria grow under diverse physical and chemical stresses.

Caenorhabditis elegans has been used as a model to investigate molecular mechanisms of bacterial virulence (Labrousse *et al*., 2000; Tan & Ausubel, 2000). Advantages such as availability of a large number of *C. elegans* mutants, the fact that it is a very simple organism, on that is easy to culture and handle, make this invertebrate a good candidate for studies on *P. shigelloides* virulence factors. There is a need to establish host-pathogen models for *P. shigelloides* that can reflect "in vivo" the interactions that, otherwise, can be only conjectured by "in vitro" conditions.

Another interesting question still to be answered is the role of migratory birds in the distribution of *P. shigelloides*. These animals might be involved in the spread of this and other enteropathogens from one geographical region to another. The importance of tracking these microorganisms is fundamental for epidemiological and ecological studies.

There is also a requirement to establish a web-based PFGE-pattern-based database that can provide information for researchers for comparison with their own material.

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Acknowledgements

I want to express my sincere gratitude to the following people:

My supervisors: Stefan B. Svenson for giving me the opportunity to start this scientific adventure. Karel Krovacek for introducing me to the aquatic bacteria world and for being there with your scientific knowledge and your support during the tough times (and we have had some, haven't we?). This thesis would not exist without you! Marie-Loiuse Danielsson-Tham, for supervising and helping me during the last part of my work.

My bosses: Jorge Moreno-Lopez, my first chief and a Spanish speaking island in this department. I also want to thank you for the delicious "c/sebiche" and lamb and those interesting and funny stories about you and your friends in different countries.
Tommy Linné, my second chief at this place. Quiet and calm like a good "Norrlänning" and very supporting every time I needed your help. Ah, and thanks for that conversation we had at the BMC. It helped me a lot.
Last, but not least, Marie-Louise Danielsson-Tham, the third in the row. Thank you for the weekly meetings and for your interest in my work and support. You have done a nice job.

My very special gratitude and respect to a person who has been an example to follow in the study of *P. shigelloides*. Thank you very much, Dr. Eva Aldova!

Department of Microbiology: Kersti (without n!!!) Larsson, Lotta (I hope you are doing fine wherever you are now), Laila M. Eriksson (thanks for your lovely work), the "Asian crew" Kui, Chai, Niwatt, Sunan and Ramon; Leif Oxburgh, Micke Berg, Martin Svenda, Micke Klingeborn, Karl Ståhl and others.

People at BMC: Magin Rovira (the Enchanter of the BMC library), Carlos Ros, Javier Martinez, Claudia Baule, Karin Ullman, Alona Pavlova, the "gang": Carles, Valerrrgggi, Elisabetta, Susanne, Arianne, Alex, Chris, Gunnar (thank you for your help and support).

People at SVA: Anna Aspan (a big kiss and hug for being such a wonderful person!), Kaggen, Göran Bölske, Stina, Carlos Concha, Karin Artursson, Helen (always having material for the courses. Thanks!), Puk, Ricardo Feinstein (muchas gracias por las estupendas fotos!)

People at KC and SLU: Violeta (gracias por tu ayuda y apoyo en los momentos difíciles), Magdalena, Manne, Claes Fellström and Jörgen.

People at the Department of Food Hygiene: Well guys, we have not had much time to get know each other and the circumstances did not help either, but I do appreciate that when I have needed something you all have been very helpful and nice to me! Thank you.

People abroad: Laura Brown (what can I say…it was a pleasure to meet you in Orihuela and the beginning of a terrific friendship. Long live the Monty Pythons!…"I didn't expect a kind of Spanish Inquisition…"), Ivan Ciznar (it was wonderful to see you in Orihuela! Do you still miss the delicious "jamon serrano"?), Anja Siitonen and Mirjami Niskanen (kiitos paljon), Laura Bravo (co-author and good friend. Gracias!), Stefano Dumontet (former PhD student at VMM, co-author and good friend. Gracie mile!), Vincenzo Pasquale (thanks for your help and for the delicious dinner! Gracie mile!) , Lili y su pequeñita (que seais muy felices!), M. Isabel (gracias por tu ayuda).

My family in Spain: My father, Natalio (this is also your thesis), my mother Maria (don't worry, I eat properly), my sister Pilar, my brother Juan Antonio, her husband "Pepe", his wife "Paquita". All my nephews and nieces (they are wonderful!)

My former family in Sweden: thank you very much for the lovely years you gave me here.

Finally, to everyone who has helped me along these years in Sweden. Thank you very much!!!

Susa, muchas gracias por tu apoyo, cariño y paciencia. Eres un sol!