Journal of Applied Microbiology 2003, **94**, 65–72

Comparison of phenotypic and genotypic markers for characterization of an outbreak of *Salmonella* serotype Havana in captive raptors

M.P. Reche, M.A. Echeita[1], J.E. García de los Rios, M.A. Usera[1], P.A. Jiménez, A.M. Rojas[2], J. Colás[3] and I. Rodriguez

Sección de Microbiología, Facultad de CC Experimentales y de la Salud, Universidad San Pablo CEU, Madrid, Spain, [1]*Laboratorio Nacional de Referencia de Salmonella, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda (Madrid), Spain.,* [2]*Bioinformatics and Biological Complexity, The Burnham Institute, La Jolla, CA, USA,* [3]*Hospital del Grupo para la Recuperación de la Fauna Salvaje (GREFA), Majadahonda (Madrid), Spain*

2002/209: received 8 May 2002, revised and accepted 9 September 2002

ABSTRACT

M.P. RECHE, M.A. ECHEITA, J.E. GARCÍA DE LOS RIOS, M.A. USERA, P.A. JIMÉNEZ, A.M. ROJAS, J. COLÁS AND I. RODRIGUEZ. 2003.

Aims: To establish a typing method for tracing the epidemic relationship of 16 strains of *Salmonella* serotype Havana isolated from captive raptors showing no symptomatology and residing in a wildlife hospital in Spain.

Methods and Results: Antimicrobial susceptibility testing, ribotyping, pulsed field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP) methodology were applied. Ten unrelated strains of serotype Havana were included as a control group to provide a basis of for the efficiency of the different markers used. All outbreak-related strains were resistant to nalidixic acid and streptomycin and showed the same ripotype, pulsotype and AFLP pattern.

Conclusions: This is the first time that AFLP analysis has been tested with serotype Havana isolates and it has demonstrated to be the most useful epidemiological tool for discriminating between unrelated and outbreak-related strains of this serotype. The results obtained suggest that all the *Salmonella* serotype Havana isolates represented a common outbreak strain whose origin of contamination could not be established although it is thought that it was the poultry meat used for raptors'diet.

Significance and Impact of the Study: Our study suggests the importance of microbiological analysis of these products in order to prevent contamination and dissemination of Salmonellae in this kind of Hospital.

Keywords: AFLP, captive raptors, outbreak, PFGE, ribotype, *Salmonella* serotype Havana.

INTRODUCTION

Salmonella enterica ssp. *enterica* serotype Havana was first reported by Corbelo and Martínez Cruz (1941) in a Cuban nursery. This serotype has been associated with poultry (Soerjadi-Liem and Cumming 1984) and its feed supplements (Bensink 1979; Yoshimura *et al*. 1979). Moreover, the pathogenicity of this serotype is well documented both in human and veterinary medicine (Makarem 1982; Mehrabian *et al*. 1988; Menon *et al*. 1994; Battisti *et al*. 1998). *Salmonella* serotype Havana isolates have also been found in the faecal microbiota of asymptomatic wild raptors and birds (Bernardo and Brandao 1996; Palmgren *et al*. 2000).

Over the last three years, 16 *Salmonella enterica* ssp. *enterica* serotype Havana isolates were obtained from faecal bacterial cultures in apparently asymptomatic captive

Correspondence to: M. Paloma Reche Sainz, Seccion de Microbiologia. Facultad de CC Experimentales y de la Salud, Universidad San Pablo CEU, Ctra de Boadilla del Monte Km 5, 600. 28668 – Boadilla del Monte (Madrid), Spain, (e-mail: preche@ceu.es).

raptors that were residents in GREFA Wildlife Hospital. Therefore, an epidemiological investigation was carried out to determine the relationship of the *Salmonella* serotype Havana isolates in order to prevent any potential spread to other animals in the hospital and to establish a molecular method for tracing the epidemic relationship among serotype Havana isolates.

Phage subtyping schemes are very useful for characterizing *Salmonella* serotypes, but since they have only been developed for those serotypes most frequently isolated, for those serotypes which are more rarely found, another typing method has to be used.

This report describes different typing methods based on phenotypic and genotypic epidemiological markers for differentiating between related and nonrelated *Salmonella* serotype Havana isolates.

The typing methods utilized in this study were the determination of antimicrobial susceptibility patterns, fingerprinting of the Ribosomal RNA Operon (rrn) loci, pulsed field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP) methodology. Ribotyping by restriction fragment length polymorphism (RFLP) analysis at the 16S ribosomal locus and PFGE are already well-established methods in molecular epidemiology and have been applied alone or in combination with other approaches for the typing of *Salmonella* serotypes. However the AFLP methodology has never been applied in epidemiological investigations into outbreaks caused by *Salmonella* serotype Havana.

MATERIALS AND METHODS

Strains

Sixteen *Salmonella* serotype Havana isolates from captive raptors used in this study are listed in Table 1. One isolate from a Black Vulture (S2); six from Kestrels (S7, S39, S79, S80, S81 and S82); two from Goshawks (S8 and S18); one from a Lesser Kestrel (S15); four from Little Owls (S19, S21, S22 and S24); one from a Montagu's Harrier (S28) and one from a Buzzard (S29).

Ten *Salmonella* serotype Havana strains from the Spanish *Salmonella enterica* collection of the Spanish National Reference Laboratory of Salmonella (SNRLS) were also included in this study and were used as a control group for the analysis of epidemiological markers. They were collected in the period from 1995 to 1999 and were selected on the basis of their different sources of isolation and geographical origins.

Antimicrobial Susceptibility Determination

Salmonella strains were tested for their susceptibility to 12 antimicrobial agents by the disk-diffusion assay, following the NCCLS guidelines (1997). The antibiotics used were ampicillin (AM), 10 μg; amoxicillin-clavulanate (AMC), 30 μg; ticarcillin (TIC), 75 μg; cefuroxime (CXM), 30 μg; ceftazidime (CAZ), 30 μg; gentamicin (GM), 10 μg; cotrimoxazole (SXT), 25 μg; nalidixic acid (NA), 30 μg; ciprofloxacin (CIP), 5 μg; chloramphenicol (C), 30 μg; streptomycin (S), 10 μg and tetracycline (TE), 30 μg (BioMérieux, Marcy l'Etoile, France).

DNA extraction

Genomic DNA was extracted from cultures of *Salmonella* by a Cetylmethylammonium Bromide (CTAB) miniprep procedure as described by Murray and Thompson (1980).

Ribotyping

Ribotyping procedure was done as described by Guerra *et al.* (1997). An aliquot of the DNA of each strain was digested with the restriction endonucleases *Pst* I or *Eco* RI following the manufacturer's instructions (Roche, Barcelona, Spain). Digoxigenin-labelled phage λHind III-digested DNA (Roche, Barcelona, Spain) was used as the molecular size marker. DNA fragments were separated by electrophoresis in D-2 agarose gels at a concentration of 0·8% (Conda, Madrid, Spain). The resulting bands were blotted onto nylon membranes (Roche, Barcelona, Spain) (Southern 1975) and the detection was done by a nonradioactive method using DIG DNA detection kit (Roche, Barcelona, Spain).

Genotyping by AFLP procedure

AFLP procedure was done as described by Valsangiacomo *et al.* (1995). The method consists of a restriction ligation reaction followed by a PCR amplification. The restriction-ligation was performed at 37 °C for 3 h in a total volume of 20 μl. The reaction mixture consisted of 2 μg of genomic DNA, 0·2 μg of each adapter-oligonucleotide [LG1, 5′-CTCGTAGACTGCGTACATGCA-3′, and LG2, 5′-tgtacgcagtctac- 3′(PE-Biosistems, Madrid, Spain)], 20 U of *Pst* I (Roche, Barcelona, Spain) and 1 U of T4 DNA ligase (Roche, Barcelona, Spain) and ligase buffer 1X, comprising 66 mM Tris/pH 7·5/, 5 mM magnesium chloride, 1 mM dithiothreitol, and 1 mM (ATP). The tagged DNA was precipitated using a 7·5-M ammonium acetate and absolute ethanol. Finally, the DNA was resuspended in 100 μl TE buffer (10 mM Tris-0·5 mM EDTA [pH 8·0]) and diluted 1 : 100 for use. The amplification was performed using Ready-To-Go PCR beads (Pharmacia Biotech, Barcelona, Spain) in a final volume of 25 μl, consisting of 1 ng template DNA, 75 ng of primer AFLP *Pst*I-G (5′-GACTGCGTACATGCAGG) (PE-Biosystems, Madrid, Spain) or primer AFLP *Pst*I-A

Table 1 Origins, antibiotic resistance, ribotypes, PFGE groups, and AFLP profiles of *Salmonella enterica* ssp. *enterica* serotype Havana strains of this study

			Ribotype		PFGE	AFLP *Pst* I	
Isolate	Source	R-pattern	*Pst* I	*Eco* RI	*Xba* I	A	G
Raptors							
S2	*Black Vulture (Aegypius monachus)*	S NA	P2	E2	X4	PA6	PG6
S7	Kestrel *(Falco tinnunculus)*	S NA	P2	E2	X4	PA6	PG6
S8	*Goshawk (Accipiter gentilis)*	S NA	P2	E2	X4	PA6	PG6
S15	Lesser Kestrel *(Falco naumanni)*	S NA	P2	E2	X4	PA6	PG6
S18	*Goshawk (Accipiter gentilis)*	S NA	P2	E2	X4	PA6	PG6
S19	Little Owl *(Athene noctua)*	S NA	P2	E2	X4	PA6	PG6
S21	Little Owl *(Athene noctua)*	S NA	P2	E2	X4	PA6	PG6
S22	Little Owl *(Athene noctua)*	S NA	P2	E2	X4	PA6	PG6
S24	Little Owl *(Athene noctua)*	S NA	P2	E2	X4	PA6	PG6
S28	Montagu's Harrier *(Circus pygargus)*	S NA	P2	E2	X4	PA6	PG6
S29	Buzzard *(Buteo buteo)*	S NA	P2	E2	X4	PA6	PG6
S39	Kestrel *(Falco naumanni)*	S NA	P2	E2	X4	PA6	PG6
S79	Kestrel *(Falco naumanni)*	S NA	P2	E2	X4	PA6	PG6
S80	Kestrel *(Falco naumanni)*	S NA	P2	E2	X4	PA6	PG6
S81	Kestrel *(Falco naumanni)*	S NA	P2	E2	X4	PA6	PG6
S82	Kestrel *(Falco naumanni)*	S NA	P2	E2	X4	PA6	PG6
	Control strains						
7961	River water	TE	P1	E1	X3	PA2	PG2
13510	Sea water	Susceptible	P1	E1	X1	PA1	PG1
13511	Sea water	Susceptible	P1	E1	X1	PA1	PG1
388	Pork	Susceptible	P2	E2	NT	PA2	PG2
390	Meat	Susceptible	P2	E2	NT	PA2	PG2
391	Beef	Susceptible	P2	E2	NT	PA2	PG2
3945	Sausage	Susceptible	P3	E3	X2	PA3	PG3
2876	Human stools	Susceptible	P1	E1	X3	PA2	PG2
4082	Human stools	Susceptible	P1	E1	X3	PA5	PG5
137	Soybeans	Susceptible	P2	E2	NT	PA4	PG4

S, streptomycin; NA, nalidixic acid; TE, tetracycline; NT, not typable.

(5′-GACTGCGTACATGCAGA) (PE-Biosystems, Madrid, Spain) and 1 mM $MgCl_2$ (total $MgCl_2$ concentration 2·5 mM). The mixture was subjected to the following amplification cycle. One cycle of 94 °C for 4 min, followed by 33 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2·5 min Amplified products (5 μl) were electrophoresed at 100 V for 4 h on 1·5% MS-8 agarose gels (Conda, Madrid, Spain) in TBE buffer (89 mM Tris, 89 mM boric

acid, 2 mM EDTA, pH 8·3) with the Molecular DNA Weight Marker X (Roche, Barcelona, Spain). The bands were visualized by staining with ethidium bromide.

Genotyping by PFGE procedure

PFGE was performed as described by Baquar *et al.* (1994). Agarose-embedded genomic DNA was digested with 15 U of restriction enzyme *Xba* I overnight at 37 °C, and DNA fragments were separated by PFGE in a 1·2% D-5 agarose gel (Conda, Madrid, Spain) using the CHEF DR- III electrophoretic apparatus (Bio-Rad, Madrid, Spain). Electrophoresis was done for 22 h with a voltage of 150 V and a linearly ramped pulse time of 1–50 s. A lambda ladder comprising 48·5 kpb concatemers (Roche, Barcelona, Spain) served as the MW markers for PFGE. The bands were visualized by staining with ethidium bromide.

RESULTS

The antibiotic resistance patterns, ribotypes, PFGE and AFLP profiles of the strains of *Salmonella* serotype Havana studied are reported in Table 1.

All the isolates from captive raptors had identical antimicrobial susceptibility patterns: susceptibility to all the antibiotics tested, except for nalidixic acid and streptomycin. The control strains were susceptible to all the antibiotics tested, except the strain 7961, which was resistant to tetracycline.

To determine the polymorphism of the *rrn* fingerprinting, Southern hybridization was carried out. The results were interpreted considering that a one-band difference was a different ribotype. The restriction endonucleases *Pst* I and *Eco* RI allowed the same degree of discrimination in the control group, yielding three ribotypes, respectively, P1 to P3 (Fig. 1) or E1 to E3 (Fig. 2). Ribotypes P1 and P2 comprised six hybridized bands and ribotype P3 comprised five hybridized bands. All the strains isolated for this study exhibited the same ribotype P2, identical to the control group strains. It consisted of six bands of hybridization ranging from 9 kb to 23 kb. The *Eco* RI ribotype E1 comprised nine bands and the ribotypes E2 and E3 comprised 10 bands of hybridization. The strains isolated for this study presented the ribotype E2. It is noteworthy that both enzymes clustered the control group and the strains of the study in the same groups.

Whole cellular DNA of the *Salmonella* serotype Havana strains was digested with *Xba* I, a rare-cutting restriction-endonuclease, and the resulting fragments were separated by pulsed-field gel electrophoresis. Three pulsotypes, X1, X2

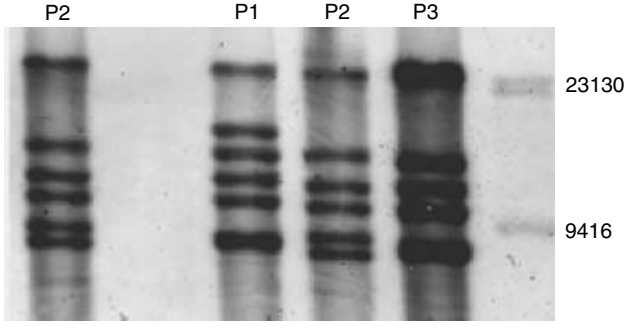

Fig. 1 Ribotypes obtained with the endonuclease *Pst* I

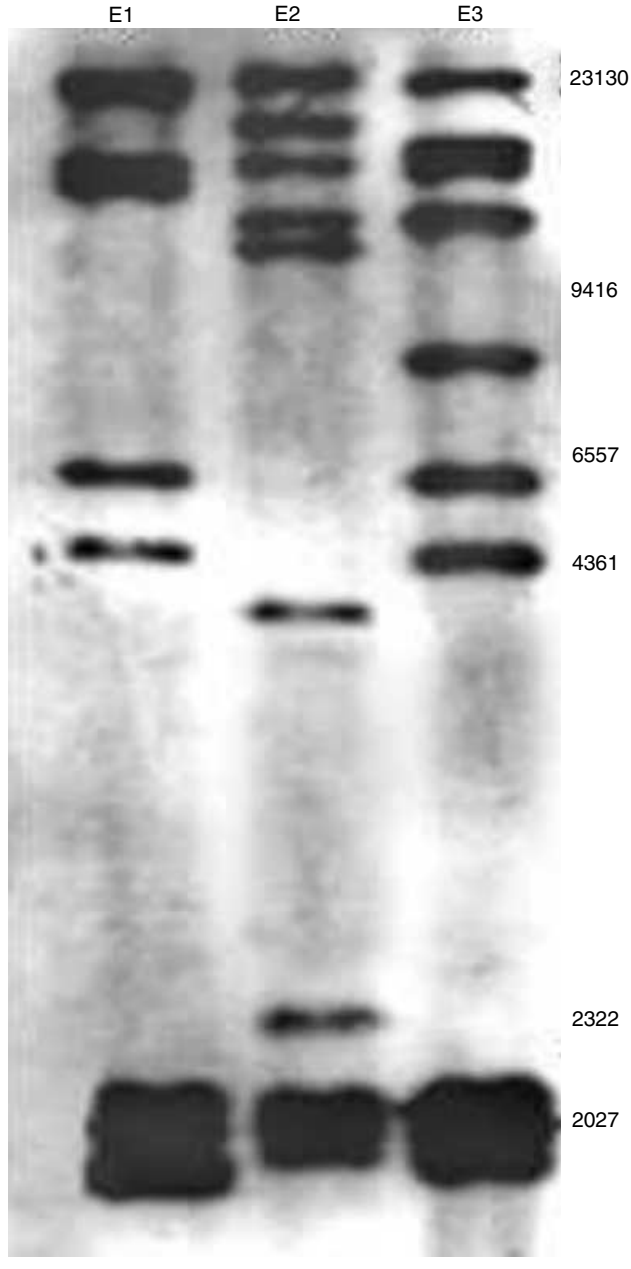

Fig. 2 Ribotypes obtained with the endonuclease *Eco* RI

and X3, could be clearly distinguished in the control group strains (Fig. 3). Profile X1, corresponding to strains 13510 and 13511, exhibited 11 restriction fragments ranging from 48 kb to 388 kb. Seven restriction fragments in the same range could be detected in pulsotype X2, corresponding to strain 3945. Strains 4082, 7961 and 2876 exhibited the profile X3, consisting of nine restriction fragments ranging from 48·5 to 321 kb.

Strains 388, 390, 391 and 137 always presented DNA degradation and were clustered as not typable (NT). To inhibit DNase activity, the Gibson *et al*. (1994) procedure, based on the utilization of formaldehyde to inactivate endogenous nuclease activity, making the samples suitable for PFGE examination, was employed. However, no good results were obtained, as is shown in Fig. 3, where the DNA degradation of strains 137, 388, 390 and 391 is noted. All outbreak-related strains were clustered together into a clonal group. All of them exhibited the same X4 pattern, different from those described above, yielding eight restriction fragments ranging from 48·5 kb to 321 kb.

Genotyping of the *Salmonella* serotype Havana strains by the AFLP-based technique described by Valsangiacomo *et al*. (1995) for *Pst* I, allowed the observation of distinct profiles of the strains from the related and unrelated individuals. Using the primer designated as primer *Pst* I-A, five different patterns could be distinguished in the control group strains, named PA1 to PA5 (Fig. 4). PA1 clustered strains 13510 and 13511, yielding eight amplification fragments. Strains 388, 390 and 391 exhibited the PA2 profile, which included seven amplification fragments. The PA3 profile corresponding to strain 3945 yielded the simplest pattern, only four amplification fragments. The PA4 pattern, corresponding to strain 137, exhibited the most complex profile, 10 amplification fragments. Finally the PA5 pattern, corresponding to strains 2876, 7961 and 4082, presented six amplification fragments. All the strains isolated for this study yielded the same pattern, PA6, different from the ones described above. It comprised six amplification fragments ranging from 300 to 1100 pb. With the primer *Pst* I-G, similar results were obtained (Fig. 5), clustering the strains in the same groups. The control group exhibited five

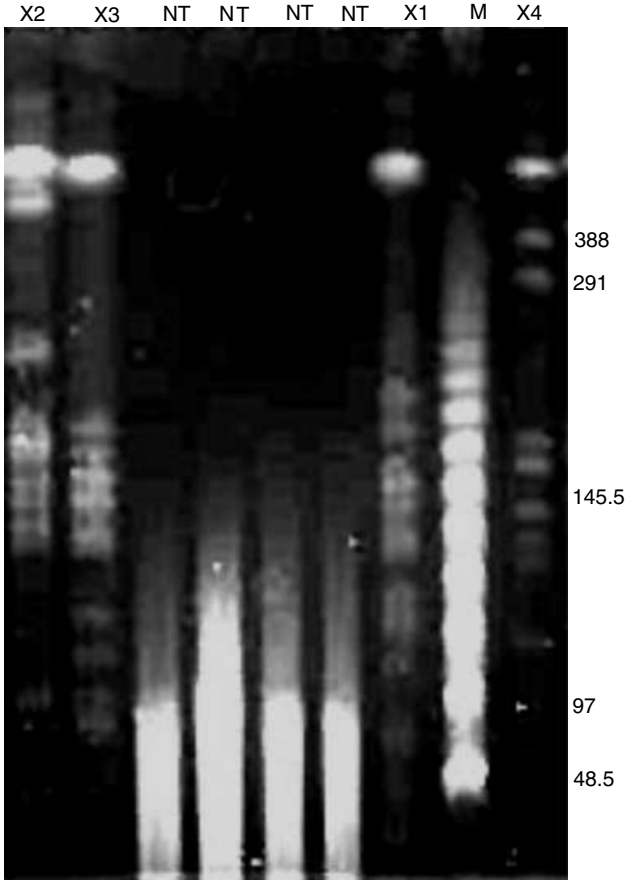

Fig. 3 PFGE patterns obtained with *Xba* I

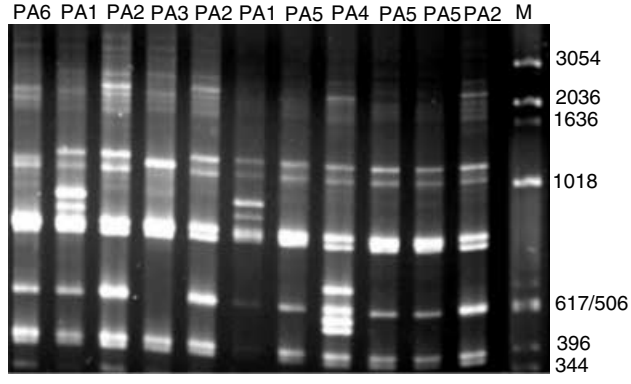

Fig. 4 AFLP patterns obtained with the primer PA

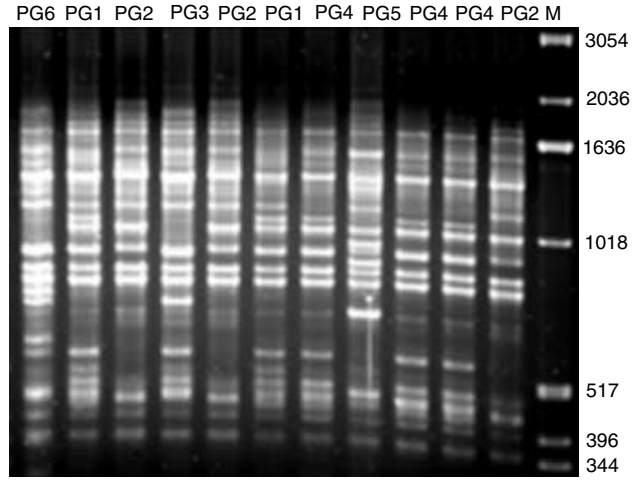

Fig. 5 AFLP patterns obtained with the primer PG

different profiles, more complex than the ones described above, named PG1 to PG5, and the strains of the study yielded a single PG6 profile that was different from the one obtained for the control group strains. In this case, all the patterns showed more than 10 amplification fragments, ranging from 300 pb to 2000 pb.

A 1400-pb sharp fragment was observed in all the patterns. The PG1, PG3, PG4 and PG6 pattern presented one amplification fragment about 600 pb, which is absent in the PG2 and PG5 profiles.

DISCUSSION

The goal of epidemiological studies is to trace the bacterial spread answering the question whether bacterial isolates collected from different sources in the same region and period are epidemiologically related and belong to the same clone. The results are applied to describe the size and nature of health problems, to investigate the causes of disease, and to evaluate interventions to treat or prevent disease (Olsen *et al*. 1993; Tenover *et al*. 1995).

In this study, 16 strains of *Salmonella* serotype Havana were isolated from the faeces of healthy captive raptors. Serotype Havana is rarely isolated in Spain and it has never been ranked there in the list of the most common serotypes isolated from human and nonhuman sources (García *et al*. 1999; Usera *et al*. 1999). However, in Iran it has been one of the most prevalent serotypes isolated from humans (Farhoudi-Moghaddam *et al*. 1990). This serotype has also been associated with raptors (Battisti *et al*. 1998).

Serotyping seems to be an useful tool to determine possible epidemiological relationships when the serotype obtained is not often found in a geographical area (Threlfall and Frost 1990). However, for further characterization of the isolates, others phenotypic and genotypic epidemiological markers were used to assess the epidemiological relationship among the strains.

Initially 10 strains of *Salmonella* serotype Havana were selected from different sources as a control group to provide a basis for the efficiency of the different markers used.

The antibiotic sensitive test showed a common pattern for all the related strains, resistance to streptomycin and nalidixic acid but, since antibiotype is an unstable character, this data is not consistent enough to assess the epidemiological relationship between the strains.

Molecular techniques commonly used for typing bacteria-ribotyping, PFGE and AFLP patterns were used to characterize isolates of serotype Havana which were epidemiologically related or unrelated. Each method used in this work was found to be efficient at discriminating the control strains from the study strains.

Ribotyping was tested with *Pst* I and *Eco* RI. These enzymes have been widely used in this type of study (Olsen *et al*. 1992; Landeras *et al*. 1997). Both endonucleases yielded the same ability for typeability and clustering the strains in the same groups. No differences could be distinguished among the strains of the study.

PFGE using the restriction enzyme *Xba* I, was also used to determine whether the 16 *Salmonella* serotype Havana isolates represented the same chromosomal outbreak strain. Indeed, this methodology has demonstrated its usefulness as an excellent technique for discriminating chromosomal differences between related and unrelated *Salmonella* strains Baquar *et al*. (1994), although it is absolutely necessary to optimize the electrophoretic conditions (Hartmann and West 1997; Usera *et al*. 1998; Kariuki *et al*. 1999).

In this case, this method posed problems because the DNA of some of the strains was very unstable, due to DNase activity. A simple method for inactivating it was used (Gibson *et al*. 1994), based on the formaldehyde fixation of the bacterial cells, with the resulting DNA in a state suitable for restriction digestion and subsequent electrophoretic analysis. However, strains 137, 388, 390 and 391 always showed DNA degradation.

A single pattern for the 16 isolates of *Salmonella* serotype Havana was observed. All these isolates represented a single related strain that have a PFGE pattern different from the epidemiologically unrelated control strains of *Salmonella* serotype Havana included in this study.

As expected, ribotyping yielded a lower number of patterns than PFGE. Several studies suggest that PFGE is the most discriminating of the available genotypic methods because it allows for the detection of minor genomic events. In fact, a good correlation between the two molecular typing methods was observed, but PFGE could discriminate isolates from the same ribotype into different subsets.

AFLP analysis was also used for fingerprinting the *Salmonella* serotype Havana isolates. In theory, this methodology provides a means of examining DNA segments distributed over the entire genome of an organism, offering this advantage over other methods that examine restriction site changes in single genes, for example, ribotyping.

Here, the utility of the AFLP-based technique described by Valsangiacomo *et al*. (1995) when applied to the differentiation of strains of *Salmonella* serotype Havana was examined. This is the first time that this method has been tested with isolates of this serotype. The endonuclease used was *Pst* I and the primer for PCR amplification were selective ones, one nucleotide extending into the restriction fragment. In this case the additional nucleotides used were A and G (Janssen *et al*. 1996). The AFLP profiles differ according to the selective primer used (Figs 4 and 5), but with both the control strains were clustered in the same groups and strains of the study yielded the same pattern.

Although PFGE usually yields good results, it can pose problems with *in situ* extraction of DNA because of DNase presence. In this case, AFLP analysis could be a good method of choice, because is less labour-intensive, easy to perform and accessible in any Microbiology laboratory.

The AFLP appeared to be an excellent tool for rapid and definitive analysis of *Salmonella* serotype Havana outbreaks. The profiles obtained were reproducible and, on visual analysis, they differentiated strains of *Salmonella* serotype Havana from related and unrelated individuals.

The results obtained in this study are interpreted to demonstrate that all the *Salmonella* serotype Havana strains isolated in this study should have a clonal origin, although the origin of the contamination could not be established. In a captive breeding centre in Rome, cases of embryonic and neonatal deaths caused by *Salmonella* serotype Havana were described (Battisti *et al.* 1998). They considered that the major source of contamination by *Salmonella* were the stocks of frozen chicks and poultry meat from avian farms. We made a microbiological screening of these products and we never cultured *Salmonella* spp. In any case, since *Salmonella* can cause morbi-mortality in raptors, it appears to be very important to make a microbiological control of all the meat used for the raptors' diet.

ACKNOWLEDGEMENTS

We are grateful to all the personnel of GREFA Wildlife Hospital for supplying the samples. This work has been supported by the grant 13/99 of the Universidad San Pablo CEU. We would like to thank Linda Hamalainen for help in preparing the manuscript.

REFERENCES

Baquar, N., Burnens, A. and Stanley, J. (1994) Comparative evaluation of molecular typing of strains from a national epidemic due to *Salmonella brandenburg* by rRNA gene and IS*200* probes and Pulsed-Field Gel Electrophoresis. *Journal of Clinical Microbiology* **32**, 1876–1880.

Battisti, A., Di Guardo, G., Agrimi, U. and Bozzano, A.I. (1998) Embryonic and neonatal mortality from salmonellosis in captive bred raptors. *Journal of Wild Diseases* **34**, 64–72.

Bensink, J.C. (1979) *Salmonella* contamination of meat and bone meal. *Australian Veterinary Journal* **55**, 13–15.

Bernardo, F.M. and Brandao, C.F. (1996) Preliminary epidemiological survey on prevalence of *Salmonella* spp. at *Bissau abattoir* (Guinea-Bissau). *Revue d'Elevage et de Médicine Vetérinaire* des. *Pays Tropicaux* **46**, 102–106.

Corbelo, A. and Martínez Cruz, J.A. (1941) Historia de la *Salmonella havana*. *Archivos de Medicina Infecciosa* **10**, 170–179.

Farhoudi-Moghaddam, A.A., Katouli, M., Jafari, A., Bahavar, M.A., Parsi, M. and Malekzadeh, F. (1990) Antimicrobial drug resistance and resistance factor transfer among clinical isolates of salmonellae in Iran. *The Scandinavian Journal of Infectious Diseases* **22**, 197–203.

García, F.N., Sanz, R.E. and Frías, N. (1999) Análisis de los serotipo de *Salmonella* aislados en 1998 por los laboratorios de sanidad ambiental de España. *Boletin Epidemiológico Semanal* **7**, 98–100.

Gibson, J.R., Southerland, K. and Owen, R.J. (1994) Inhibition of DNAse activity in PFGE analysis of DNA from *Campylobacter jejuni*. *Letters in Applied Microbiology* **19**, 357–358.

Guerra, B., Landeras, E., González-Hevia, M.A. and Mendoza, M.C. (1997) A three-way ribotyping scheme for Salmonella serotype Typhimurium and its usefulness for phylogenetic and epidemiological purposes. *Journal of Medical Microbiology* **46**, 307–313.

Hartmann, F.A.H. and West, S.E.H. (1997) Utilization of both phenotypic and molecular analyses to investigate an outbreak of multidrug-resistant *Salmonella anatum* in horses. *Canadian Journal of Veterinary Research* **61**, 173–181.

Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M. and Kersters, K. (1996) Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiology* **142**, 1881–1893.

Kariuki, S., Gilks, C., Kimari, J., Muyodi, J., Waiyaki, P. and Hart, C.A. (1999) Analysis of *Salmonella enterica* serotype Typhimurium by phage typing, antimicrobial susceptibility and pulsed-field gel electrophoresis. *Journal of Medical Microbiology* **48**, 1037–1042.

Landeras, E., Usera, M.A., Calderón, C. and Mendoza, M.C. (1997) Usefulness of phage typing and 'two-way ribotyping' to differentiate *Samonella enteritidis* strains. *Microbiología SEM* **13**, 471–480.

Makarem, E.H. (1982) Outbreak of a multiple drug resistant *Salmonella havana* originating in pediatric wards of two hospitals in Shiraz, Iran. In vitro susceptibility patterns. *Journal of Tropical Pediatry* **28**, 14–19.

Mehrabian, S., Ghorishi, B. and Madjid, S. (1988) Observation on the incidence of non typhoidal *Salmonella havana* drug resistance and pathogenic strength to the children of under one year old age. Iran 1976–86. *Iranian Journal of Public Health* **17**, 81.

Menon, P.K., Raguraman, T.S., Chaudhury, M., Kudesia, S. and Rao, D.V. (1994) Nenonatal Salmonella havana meningitis. *Indian Journal of Pathology and Microbiology* **37**, 435–438.

Murray, M.G. and Thompson, W.F. (1980) Preparation of genomic from bacteria. In: *Current Protocols in Molecular Biology*. Vol 1. eds Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. pp. 2.4.1–2.4.5 New York: Greene Publishing Associates and Wiley-Interscience.

National Committee for Clinical Laboratory Standards. (1997) Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically. Approved standard M2–A5. Wayne, PA: National Committee for Clinical Laboratory Standards.

Olsen, J.E., Brown, D.J., Baggesen, D.L. and Bisgaard, M. (1992) Biochemical and molecular characterization of *Salmonella enterica* serovar Berta, and comparison of methods for typing. *Epidemiology and Infection* **108**, 243–260.

Olsen, J.E., Brown, D.J., Skow, M.N. and Christensen, J.P. (1993) Bacterial typing methods suitable for epidemiological analysis. Applications in investigations of salmonellosis among livestock. *Veterinary Quarterly* **15**, 125–135.

Palmgren, H., McCafferty, D., Aspan, A., Broman, T., Sellin, M., Wollin, R., Bergstrom, S. and Olsen, B. (2000) Salmonella in

sub-Antarctica: low heterogenecity in *Salmonella* serotypes in South Georgian seals and birds. *Epidemiology and Infection* **125**, 257–262.

Soerjadi-Liem, A.S. and Cumming, R.B. (1984) Studies of the incidence of Salmonella carriers in broiler flocks entering a poultry processing plant in Australia. *Poultry Science* **63**, 892–895.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Mololecular Biology* **98**, 503–517.

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H. and Swaminathan, B. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology* **33**, 2233–2239.

Threlfall, E.J. and Frost, J.A. (1990) The identification, typing and fingerprinting of *Salmonella*: laboratory aspects and epidemiological application. *Journal of Applied Bacteriology* **68**, 16.

Usera, M.A., Aladueña, A., Díez, R., de la Fuente, M., Gutiérrez, R., Cerdán, P. and Echeita, A. (1999) Análisis de las cepas de *Salmonella* sp. aisladas de muestras clínicas de origen humano en España en el año 1998. *Boletin Epidemiológico Semanal* **7**, 45–48.

Usera, M.A., Rodriguez, A., Echeita, A. and Cano, R. (1998) Multiple analysis of a foodborne outbreak caused by infant formula contaminated by an atypical *Salmonella virchow* strain. *European Journal of Clinical Microbiology and Infectious Diseases* **17**, 551–555.

Valsangiacomo, C., Baggi, F., Gaia, V., Balmelli, T., Peduzzi, R. and Piffaretti, J.C. (1995) Use of amplified fragment length polymorphism in molecular typing of *Legionella pneumophila* and application to epidemiological studies. *Journal of Clinical Microbiology* **33**, 1716–1719.

Yoshimura, H., Nakamura, H. and Sato, S. (1979) Incidence of salmonellae in animal feed ingredients in Japan. *National Institute of Animal Health Quarterly* **19**, 107–113.