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Salmonella Indiana as a cause of abortion in ewes: 
genetic diversity and resistance patterns

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Abstract

Salmonella enterica subspecies enterica Indiana, a food-borne serovar uncommon in most countries, was responsible for an outbreak of abortion in a flock of Lacaune dairy ewes in southern Spain. Drinking water and feedstuff samples were analysed in an attempt to determine the source of the infection. Pigeons (Columba livia) and turtledoves (Streptopelia turtur) in close contact with the ewes were captured and examined for the bacterium.

Seventeen S. Indiana strains were isolated from the ewes and wild birds and the genetic similarity among them analysed by PFGE after the digestion of their genomic DNA with the restriction enzyme XbaI. The results suggest the wild birds might be responsible for the outbreak in the ewes. The strains recovered were fully susceptible to 15 out of the 16 antimicrobial agents tested: ampicillin, amoxycillin clavulanate, cephalothin, ceftriaxone, gentamicin, neomycin, streptomycin, tetracycline, ciprofloxacin, enrofloxacin, sulphonamides, trimethoprim-sulphamethoxazole, apramycin, colistin and chloramphenicol. Differences in the resistance pattern to nalidixic acid were observed; 11 strains (64.7%) were nalidixic acid resistant (R-Nx) and 6 (35.3%) sensitive (S-Nx). Among the R-Nx strains, a substitution of Gly to Cys at position 81 (Gly81âCys) of the gyrA gene in 10 strains isolated from wild birds and ovine foetuses, and of Asp to Tyr at position 87 (Asp87âTyr) in one strain isolated from ewe faeces, were revealed by sequencing the gene.

To control the outbreak, enrofloxacin treatment was administered for five days. The same therapy was used to prevent infection during following gestation cycles, administering the antimicrobial agent at presentation and over four weeks before birth. Anti-bird meshes and closed drinking and feeding troughs were also installed to prevent further contact of the ewes with wild birds.

Keywords: S. Indiana; ewes; abortion; PFGE; antimicrobial susceptibility; gene gyrA
1. Introduction

The genus *Salmonella* is the largest and most heterogeneous group of the medically important Gram negative bacteria. The genus has two species, *S. enterica* divided in 6 subspecies and consisting of more than 2500 serovars, and *S. bongori* (Popoff et al., 2004). Some serovars have been associated with specific animal reservoirs, while others are known to infect a wide variety of animals including humans (Davies, 2004). Salmonellosis can represent a serious occupational and public health hazard. The problem of salmonellosis is compounded by an increase in the emergence of antimicrobial resistance in isolates from animal feed (Usera et al., 2002). *Salmonella* is usually transmitted via faecal-oral route and by direct contact with infected animals or contaminated feed and water, although it has been suggested that wild carrion-eating birds and rodents act as reservoirs of infection (Pennycot et al., 2006).

Ovine salmonellosis is manifested by a range of clinical signs of variable severity, mainly depending on the serovar involved. Of all the *Salmonella* serovars described, Abortusovis is that most frequently associated with ovine salmonellosis, especially in Europe and the Middle East where it commonly causes ewes to abort (Beuzon et al., 1997; Valdezate et al., 2007). *Salmonella enterica* serovar Indiana, which was first described in 1955, isolated from a 9-month-old girl, in Indianapolis (Price and Carter, 1967), is a serovar frequently isolated from poultry and ducks (Fuzihara et al., 2000; Roy et al., 2002; Tsai and Hsiang, 2005; Skov et al., 2004). In humans it is associated with gastroenteritis and other sporadic disorders such as miscarriage (Beckers et al., 1982; Punia et al., 1998; Mason et al., 2001). However, its relationship with animal disease is limited (Agnoletti et al., 1999; SAC, 2005). Actually, there is no phage typing scheme for *S. Indiana*, and DNA fingerprinting methods for strain subdivision are necessary. So far, Pulsed Field Gel Electrophoresis (PFGE) profiles have been performed as molecular characterization techniques with success to identify different and predominant Indiana genotypes (Punia et al., 1998).

The present work describes an outbreak of abortion due to *Salmonella* Indiana in a flock of sheep in southern Spain. An epidemiological survey was undertaken to determine the possible origin of the infection. PFGE and antimicrobial susceptibility methods were used to characterise the Indiana genotypes and the antimicrobial resistance phenotypes of the strains.
2. Materials and Methods

In September 2006 an outbreak of abortion occurred in a flock of Lacaune sheep in southern Spain (Dos Torres, Province of Cordoba). The farm was composed of several gestation units, lamb raising units, and a sick bay. The affected pregnancy unit had approximately 150 ewes, of which 24 were affected (16%). Clinical signs included epidemic abortion during the last-third of pregnancy, stillbirths, and premature and non-viable lambs. Numerous pigeons and turtledoves were observed during visits made to the farm. These birds had contact with the sheep, their feed and water.

2.1. Isolation and identification procedures

Aborted foetuses, material from dead lambs (liver and gastric contents), placentas and vaginal swabs were taken for examination. Blood samples from aborted ewes were analysed microbiologically and serologically at the Animal Health Department of the Veterinary Faculty, University of Cordoba, to identify the pathogen responsible.

Conventional microbiological and serological methods were carried out to clarify the possible causal agents associated to this abortion outbreak. To confirm the presence of Salmonella, cultures were grown directly on xylose lysine decarboxylase (XLD) agar (Oxoid, Ltd., Madrid, Spain). Before use, all plates were incubated for 48 h at 37°C under aerobic conditions. A pool of faecal material from the gestation unit was also analysed by inoculating 25g into 225 ml of buffered peptone water (Oxoid, Spain) (pre-enrichment at 37°C for 18-24 hours) and then transferring 0.1 ml of this solution after 24 h into 10 ml Rappaport-Vassiliadis (RV) broth (Oxoid, Spain) (enriched for 24-48 hours at 42°C). Swab samples from the RV broth were plated on XLD agar.

Feed from the pregnancy unit was cultured as for faeces. The drinking water was directly cultured on McConkey agar, XLD agar, blood agar, Columbia blood agar and Sabouraud dextrose agar (Oxoid, Spain) to detect possible pathogens.

Six wild birds (4 pigeons and 2 turtledoves) were captured and euthanised. After post mortem examination, liver, duodenum and caecum samples were directly cultured on XLD medium (37°C, 24 h).

Presumptive Salmonella colonies were biochemically confirmed as such using lysine iron agar (Difco, BD, Spain), Kligler’s iron agar (Oxoid, Spain) and motility indole ornithine agar (Difco, BD, Spain). Salmonella enterica isolates were serotyped by the slide agglutination method using commercial antisera (Bio-Rad, Spain; Statens Serum Institut, Izasa, Spain).
2.2. Pulsed field gel electrophoresis

PFGE was performed following the PulseNet-Europe protocol (http://www.pulsenet-europe.org/docs.htm). Total DNA was digested with XbaI (Roche Applied Science), and the obtained fragments were separated in 1% agarose (Seakem gold Agarose, Iberlabo Spain) gels using the CHEF-DR-II system (Biorad Laboratories Inc. Hercules, CA, USA). Electrophoresis was carried out with 0.5X TBE buffer at 6V/cm and 14°C. The running time was 21h and the pulse ramp time was 2.2-63.8s. The XbaI digested DNA from *S. enterica* Braenderup H9812 was used as a molecular size marker. Pattern clustering was performed using UPGMA (unweighted pair-group method with an arithmetic mean) and the Dice coefficient (Nei and Li, 1979) with a tolerance index of 0.5%. Fragments smaller than 30kb were disregarded according to the PulseNet guidelines for standardization.

2.3. Antimicrobial susceptibility

All strains were screened for resistance to 16 antimicrobial agents on Mueller-Hinton agar (Oxoid, Spain) using the disk diffusion method: ampicillin (10 µg/disk), amoxycillin clavulanate (20 and 10 µg/disk), cephalothin (30 µg/disk), ceftriaxone (30 µg/disk), gentamicin (10 µg/disk), neomycin (30 µg/disk), streptomycin (10 µg/disk), tetracycline (30 µg/disk), ciprofloxacin (5 µg/disk), enrofloxacin (5 µg/disk), sulphonamide compounds (200 µg/disk), trimethoprim-sulphamethoxazole (1.25 and 23.75 µg/disk), apramycin (15 µg/disk), nalidixic acid (30 µg/disk), colistin (50 µg/disk) and chloramphenicol (30 µg/disk). All antimicrobial agents were purchased from Oxoid (Oxoid, Spain). *Escherichia coli* ATCC 25922 was used as a control. The measurement and interpretation of growth inhibition diameters was assessed following guidelines for veterinary antimicrobial susceptibility tests (CLSI, 2002).

2.3. DNA extraction, gyrA amplification and sequencing.

The QRDR of *gyrA* gene was amplified with primers *gyrA1* (5'-GGTACACCGTGCCGTACTTT-3') and *gyrA2* (5'-TCCACGAAATCCACCAGTC-3') as described by Griggs and others (1996). Five microliters of a strain suspension boiled for 10 min and briefly centrifuged was used as DNA template. The PCR was performed using the PureTaq Ready-to-Go PCR beads system (GE Healthcare Life Sciences, UK). PCR amplification was as follows: denaturation at 95°C for 5 min; 30 cycles of 95°C for 30 s; 55°C for 30 s and 72°C for 30 s; and a final extension cycle at 72°C for 7 min. The amplification products were purified with the GFX PCR DNA and Gel Band
Purification Kit (GE Healthcare Life Sciences, UK) and sequenced by using the same primers. Sequencing was performed on an ABI PRISM3700 DNA analyzer (Applied Biosystems, Applera Hispania, S.A. Spain) and the Taq Dye Deoxy terminator cycle sequencing kit (Applied Biosystem/Perkin–Elmer). The gyrA sequences were aligned and compared to that previously deposited in GenBank with accession number X78977 and sequences analysis was performed with the Lasergene v.5.0 software (DNA-star, Madison WI, USA).

2.4. Nucleotide sequence accession numbers.

The GenBank accession numbers for gyrA partial sequences of the quinolone-resistant strains are: EU929063 for point mutation Gly81àCys and EU929064, for point mutation Asp87àTyr.

3. Results and Discussion

Abortion and mortality among newborn lambs cause severe economic problems in areas with sheep-based economies. S. Abortusovis is the main agent responsible for abortion in ewes, although S. Typhimurium and S. Dublin can also cause reproductive failure (Intorre et al., 2005; Habrun et al., 2006; Valdezate et al., 2007). This paper describes an outbreak of abortion during the last third of gestation in a flock of Lacaune ewes caused by S. enterica serovar Indiana, with lethality rates close to 16%. Other problems included stillbirths and premature and non-viable lambs without other clinical disorders.

Nine Salmonella isolates belonging to the Indiana serovar (1,4,12:z:1,7) were recovered (in pure culture) in moderate to high numbers from the pathological samples (vaginal swabs, liver or stomach contents of aborted foetuses, non-viable lambs, and faeces). Other possible causes of the outbreak, such as Brucella, Campylobacter, Chlamydophila, Coxiella, Listeria, Leptospira and Toxoplasma were excluded by microbiological and serological testing.

The worldwide frequency of the isolation of S. Indiana is low. It is isolated from domestic poultry and ducks (Fuzihara et al., 2000; Roy et al., 2002; Skov et al., 2004) but is also responsible for enteric and other sporadic disorders in humans (Baxter and Morton, 1987; Bouche et al., 1993; Peluchon et al., 1997; Punia et al, 1998; Mason et al., 2001). The clinical repercussions for animal species are usually very limited, although an outbreak characterised by death and abortion in a commercial flock of ewes in the Edinburgh area has been described (SAC VS, 2005). The present report is only the second description of an outbreak of salmonellosis in sheep due to S. Indiana.
In the present work, the feed and drinking water of the affected sheep were negative for *Salmonella* - unexpected results given that the oral route is the main route of infection described for this pathogen (Valdezate et al., 2007). The present results agree with those of previous studies that suggest sources other than feed or drinking water spread the bacterium (Agnoletti et al., 1999), for example, it is known that litter beetles act as reservoirs of *Salmonella* Indiana in broiler chicken rearing facilities (Skov et al., 2004). Eight *Salmonella* Indiana strains were isolated from the liver and intestinal samples of the pigeons and turtledoves. 17 S. Indiana strains isolated from the ovine and avian samples were analysed by PFGE (Fig. 1). The number of bands originated ranged from 14 to 15 with two different PFGE profiles (SIndX1 and SIndX2). PFGE patterns analysis revealed a high similarity among pulsotypes with just one different band, confirming the genetic relationships between the sheep and wild bird strains. These results show that pigeons and turtledoves could have been the source of the present outbreak, probably by faecal contamination of the pregnancy unit environment. These data confirm the important carrier role of wild birds for the maintenance and circulation of pathogens among herds (Davies, 1997; Davies and Breslin, 2001; Lillehaug et al., 2005; Pennycott et al., 2006).

All the isolated strains were fully susceptible to the majority of the antimicrobial agents tested, but differences in resistance to nalidixic acid were observed (Figs. 1 and 2). Eleven of the 17 isolates (64.7%) were nalidixic acid resistant (R-Nx) and six (35.3%) were susceptible (S-Nx); representatives of both types were isolated from wild birds and ovine samples. Among the R-Nx strains, point mutations of Gly to Cys at position 81 (in 10 strains isolated from wild birds and the ovine foetuses) and Asp to Tyr at position 87 (in the strain isolated from ewe faeces) were revealed by *gyrA* gene sequencing. The finding of more than one *Salmonella* Indiana resistance pattern indicates that pigeons and turtledoves may harbour different strains that could be shed simultaneously.

Treatment with enrofloxacin for five consecutive days successfully controlled this outbreak. The same therapy was used to prevent infection during following gestation cycles, administering the antimicrobial agent at presentation of the male and over four weeks before birth. Other preventive measures such as anti-bird mesh systems and closed drinking and feeding troughs are now used at the farm to avoid contamination from wild birds.
In conclusion, the present work shows: (i) *Salmonella* Indiana causes abortions in ewes, with carrier wild birds as the probable source of infection; (ii) the coexistence in the birds of nalidixic-acid susceptible *S*. Indiana strains and strains that were resistant as a result of single point mutations in *gyrA* leading to the Gly81→Cys substitution.

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Figure 1. Phylogenetic relationships among the 17 strains of *Salmonella enterica* serovar Indiana detected by PFGE, and the associated nalidixic acid-resistant types.
Fig. 1. Phylogenetic relationships among 17 strains of *Salmonella enterica* serotype Indiana and the resistotypes detected. The two pulsotypes identified are listed adjacent to the corresponding XbaI-PFGE banding patterns. The DNA molecular weight scale was provided by the Braenderup Salm reference control. $R^{A-Nx}$, *gyrA* substitution Gly81Cys; $R^{B-Nx}$, *gyrA* substitution Asp87Tyr.