ORIGINAL ARTICLE 10.1111/j.1469-0691.2006.01468.x

O148 Shiga toxin-producing *Escherichia coli* outbreak: microbiological investigation as a useful complement to epidemiological investigation

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ABSTRACT

An outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O148 infection occurred among wedding attendees in France in June 2002. A retrospective cohort study was performed and ten cases were identified, including two adults with haemolytic uraemic syndrome (HUS). The analytical study revealed that >80% of affected individuals had eaten lightly roasted mutton and poultry pâté, but only the consumption of pâté tended to be associated with illness (relative risk 3.4; 95% CI 0.8–14.4). Left-overs (cooked mutton and raw offal) and processed foods (pâté) from the same batches as served at the party were sampled. Human, food and environmental samples were examined for the Shiga toxin (*stx*) gene and virulence traits by PCR. *Stx*-positive samples were cultured for STEC. HUS cases were tested for serum antibodies against 26 major STEC serogroups. An STEC O26 strain (*stx1*, *eae*, *ehxA*) was isolated from one case with diarrhoea, and an STEC O148 strain (*stx2c*) from one case of HUS. Serum antibodies against O26 were not detected in either of these patients; antibodies against O148 were not tested. Three STEC strains were isolated from the mutton and the offal (*stx2c*, O148), and two from the pâté (*stx2c*, O-X and O-Y). The isolates from the mutton were indistinguishable from the human *stx2c* isolate, whereas the pâté isolates differed. Although four different STEC strains were identified in patients and foods, the results of molecular subtyping, in conjunction with analysis of food consumption patterns, strongly suggested that this outbreak was caused by mutton contaminated with STEC O148.

Keywords *Escherichia coli*, foodborne infection, molecular typing, outbreak, Shiga toxin, STEC

Original Submission: 16 May 2005; **Revised Submission:** 15 November 2005; **Accepted:** 15 February 2006

Clin Microbiol Infect 2006; 12: 992–998

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC), especially *E. coli* O157:H7, are an important cause of foodborne disease in industrialised countries. Clinical manifestations of infection with STEC range from mild diarrhoea to severe and specific complications, such as haemolytic uraemic syndrome (HUS) [1]. The main natural reservoir of STEC is ruminants, especially cattle. Human infections are mainly food-related, but person-to-person and direct contact with contaminated cattle or goats have also been identified as transmission routes [2]. Several outbreaks of STEC and *E. coli* O157 infection in industrialised countries have been associated with various food sources, including undercooked ground beef, unpasteurised milk products, raw vegetables, unpasteurised cider and water [3].

In France, surveillance of STEC infections is based on nationwide surveillance for HUS in children aged <15 years [4]. Since 1996, the annual incidence of HUS has been stable in France (mean 0.74/100 000 children aged <15 years), with most cases being sporadic and caused by *E. coli* O157 infection [5]. During the last 10 years, only three outbreaks of infection

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have been detected [6–8]. This report describes an outbreak investigation that illustrates the combined use of epidemiological and microbiological methods to identify the source of a non-O157 STEC outbreak.

MATERIALS AND METHODS

On 10 July 2002, the hospitalisation of two adults with HUS was notified to the national public health authority (Institut de Veille Sanitaire). Both individuals had attended a wedding party, 2 days before the onset of illness. Several other wedding attendees were reported to have suffered from gastroenteritis. An investigation was initiated to determine the extent of the outbreak and the source of infection.

Epidemiological and trace-back investigations

A retrospective cohort study was conducted to identify wedding attendees with diarrhoea (more than three stools per day for at least 1 day) during the 8-day period following the party on 29 June 2002. All guests were interviewed by telephone to determine the food and drinks consumed during the party. The suspected foods were traced to identify the supply channels of the retail outlets and the origin of the food. Since one of the suspected foods was distributed nationally, all French nephrology hospital departments were asked to notify all cases of HUS in June and July in order to identify any increase in HUS cases or cases related to the suspected food.

Party attendees, food and animal sampling

Stool specimens were taken from cases of diarrhoea, and serum specimens were also taken from the HUS cases. Leftovers and suspected food from the same batches that were served at the party (when no food remained to be sampled) were sampled. At the farm where the animals were slaughtered, the faeces from other animals, water from the drinking trough and feed were also sampled. All human, food and animal samples were sent to the relevant reference laboratories (the National Reference Laboratory for *E. coli* and *Shigella* and the National Veterinary School) for immediate processing and analysis by culture and PCR. Human stool samples were examined for *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia* spp. and STEC.

Isolation and detection of STEC from stool samples

A single swab was obtained from each patient and placed in transport medium. Swabs were plated on trypticase soy agar, Drigalski agar and MacConkey agar (Oxoid, Dardilly, France). After overnight growth at 37°C, between ten and 50 lactose-positive colonies were chosen from each sample and identified as *E. coli* by conventional biochemical methods or by amplification of the β-glucuronidase gene (*uidA*) [9]. The *E. coli* isolates were stored at −80°C on glass beads in glycerol 50% w/v in brain–heart infusion broth (Difco, Detroit, MI, USA).

One loopful of each isolated colony was suspended in 1 mL of sterile water and then centrifuged to pellet the cells. DNA was extracted with InstaGene Matrix (Bio-Rad, Marnes-le-Coquette, France). Primers LIN5′ and LIN3′ were used for the amplification of all members and variants of the *stx* gene family [10]. Subtyping of *stx2* genes was performed by *Hin*cII digestion of the 900-bp DNA product, and by *Hae*III and *Pvu*II digestion of the 348-bp DNA product, both as described previously [11,12]. The *eae* gene was detected using primer pairs fM1 and rYu4 as described by Beaudry *et al*. [13], and the *ehxA* gene was detected using primer pairs hlyA1 and hlyA4 as described by Schmidt *et al*. [14] (Table 1). Reference *E. coli* strains used as controls were EDL933 (O157:H7, *stx1*, *stx2*, *ehxA*, *eae*), H19 (O26:H11, *stx1*, *eae*), E32511 (*stx2*, *stx2c*, *eae*), HB101 (negative control), and 2348/69 (*eae*) [11].

Isolation and detection of STEC from food, animal and environmental samples

Portions (25 g) of each food and animal sample were placed aseptically in a stomacher bag with 225 mL of buffered peptone water (bioMérieux, Marcy l'Etoile, France), mixed using a stomacher apparatus, and incubated at 37°C overnight.

Table 1. Primer sequences used for detection of the *stx*, *stx*$_1$, *stx*$_2$, *eae*, *ehxA* and *uid*A genes

Gene	Primer	Sequence	PCR product size (bp)	Reference
Stool samples				
*uid*A	UAL	AAA ACG GCA AGA AAA AGC AG	147	[9]
	UAR	ACG CGT GGT TAC AGT CTT GCG		
stx	LIN5′	GAA CGA AAT AAT TTA TAT GT	900	[10]
	LIN3′	TTT GAT TGT TAC AGT CAT		
eae	FM1	CAT TAT GGA ACG GCA GAG GT	790	[13]
	RYu4	ATC TTC TGC GTA CTG CGT TCA		
ehxA	hlyA1	GGT GCA GCA GAA AAA GTT GTA G	1551	[14]
	hlyA4	TCT CGC CTG ATA GTG TTT GGT A		
Food, animal and environmental samples				
*uid*A	PT2	GCG AAA ACT GTG GAA TTG GG	252	[16]
	PT3	TGA TGC TCC ATC ACT TCC TG		
stx	ES149	CGA AAT (CT)C(CT) CTC TGT AT(CT) TG(CT) C	323	[15]
	ES151	GA(AG) C(AG)A AAT AAT TTA TAT GT		
stx1	LP30	CAG TTA ATG TGG TGG CGA AGG	348	[16]
	LP31	CAC CAG ACA ATG TAA CCG CTG		
stx2	LP43	ATC CTA TTC CCG GGA GTT TAC G	584	
	LP44	GCG TCA TCG TAT ACA CAG GAG C		
eae	EAEP1	CTG AAC GGC GAT TAC GCG AA	917	[17]
	EAEP2	CCA GAC GAT ACG ATC CAG		
ehxA	hlyAF	GCA TCA TCA AGC GTA CGT TCC	534	[18]
	hlyAR	AAT GAG CCA AGC TGG TTA AGC T		

Synthetic probes. *E. coli* O103:H2 possessing only the stx_1 gene (provided by the Institut Pasteur, Lille, France) and *E. coli* O157:H7 possessing only the stx_2 gene (ATCC 43895) were used to prepare stx_1 and stx_2 DNA probes, respectively. The DNA from each strain was extracted with phenol–chloroform, and PCR for *stx* genes was performed with degenerate primers ES149 and ES151, as described by Read *et al*. [15] (Table 1). PCR detection of the O157:H7 *uidA* gene was performed on the *stx*-positive samples with primers PT2 and PT3 as described by Cebula *et al*. [16] (Table 1).

The PCR fragments were separated on and excised from agarose gels, and purified using the Geneclean II kit (QBiogen, Heidelberg, Germany). Digoxigenin labelling was performed with the DIG-High Prime kit (Boehringer, Mannheim, Germany) as recommended by the manufacturer. In brief, DNA (*c.* 1 μg) was first denatured by heating in a boiling bath for 10 min and chilling quickly in an ice–ethanol bath. Hexanucleotide primers, nucleotides (dATP, dTTP, dGTP, dCTP and digoxigenin-11-dUTP) and Klenow enzyme were then added, giving a final volume of 20 μL. Labelling took place at 37°C overnight. The reaction was stopped by heating to 65°C for 10 min. The labelled probes were stored at −20°C.

Colony hybridisation. Enriched samples that gave a positive result with the *stx* PCR were processed as follows: 100 μL of the 10^{-4} and 10^{-5} dilutions of each enriched sample were spread on MacConkey agar plates (bioMérieux). After overnight incubation at 37°C, the dilution with the highest number of isolated colonies was selected for colony hybridisation and was cooled at 4°C for 30 min. A nylon membrane disk (Boehringer) was placed on the surface of the plate for 10 min, then on blotting paper (Schleicher & Schuell, Dassel, Germany) soaked with denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 10 min, and then on paper soaked with neutralisation solution (1.5 M NaCL, 1 M Tris-HCl, pH 7.4) for 15 min. Finally, the disk was placed on blotting paper soaked with 2× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0) for 10 min, dried, and then baked for 90 min at 80°C. The colony lift was then treated with 1 mL of proteinase K 2 mg/mL (Merck, Darmstadt, Germany) to remove cell debris, and washed vigorously with 2× SSC for 15 min. Prehybridisation, hybridisation (with a hybridisation solution containing 100 ng of each labelled probe) and immunological detection were performed with the DIG Nucleic Acid Detection Kit (Boehringer), following the manufacturer's instructions. The STEC isolates corresponding to positive hybridisation signals were picked from the hybridisation plate and grown overnight in nutrient broth. The isolates were confirmed as *E. coli* with the API 20E system (bioMérieux) and were examined for the presence of the stx_1, stx_2, *eae* and *ehxA* genes as described previously [16–18] (Table 1).

Serogrouping of all human, food, animal and environmental STEC isolates

Serogrouping was performed by standard procedures with commercial antisera raised against *E. coli* somatic (O) antigens; sera were obtained from Bio-Rad, Eurobio (Les Ulis, France) or Statens Serum Institut (Copenhagen, Denmark). These tests were able to identify the following 42 serogroups: O1, O6, O8, O15, O18, O20, O25, O26, O27, O28ac, O29, O44, O55, O63, O78, O86a, O111, O112ac, O114, O115, O119, O124, O125, O126, O127a, O128, O136, O142, O143, O144, O146, O151, O152, O153, O157, O158, O159, O164, O166, O167, O168 and O169. When serogrouping was negative, isolates were investigated for their O types by PCR amplification of the O-antigen gene cluster [19], followed by restriction enzyme cleavage of PCR products with *Mbo*II. Restriction fragment length polymorphism patterns were analysed using Taxotron software [20] and a database containing the restriction fragment length polymorphism patterns of reference strains [19]. The O antigen of suspected strains was confirmed at the WHO International *Escherichia* and *Klebsiella* Centre (Statens Serum Institut).

Ribotyping of all human, food, animal and environmental STEC isolates

Automated ribotyping was performed using the RiboPrinter Microbial Characterization System (Qualicon, Wilmington, DE, USA) according to the manufacturer's instructions. Digestion by restriction endonuclease *Mlu*I, gel separation, transfer and hybridisation with a chemiluminescent-labelled DNA probe containing the *E. coli* rRNA operon, were completed within 8 h. Gel images were analysed and compared using the Taxotron package [20].

Pulsed-field gel electrophoresis (PFGE) of all human, food, animal and environmental STEC isolates

Each isolate was grown overnight at 37°C on trypticase soy agar. Several colonies were resuspended in 10× TE buffer (100 mM Tris, 10 mM EDTA, pH 8.0) to OD_{600} 1.6–1.8. DNA plugs were prepared using standard PFGE procedures. Digestion of genomic DNA embedded in plugs was carried out using 30 U of *Xba*I (Amersham Pharmacia Biotech, Uppsala, Sweden) at 37°C overnight. PFGE was performed using the CHEF DR III system (Bio-Rad) in SeaPlaque GTG agarose (BMA, Rockland, ME, USA) 1% w/v gels in 0.5× TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.2) at 6 V/cm for 24 h at 14°C, with a ramped pulse time of 7–12 s for 11 h, followed by 20–40 s for 13 h, and an electric field angle of 120°. Multimers of phage lambda, Low Range Size Marker (New Englands Biolabs, Beverly, MA, USA) and *Salmonella enterica* serovar Branderup DNA digested with *Xba*I were used as molecular size standards. After electrophoresis, the gels were stained with ethidium bromide or SYBR Green (BMA) (1 mg/L) for 30 min and photographed under UV light. The PFGE patterns obtained were analysed and compared using the Taxotron package [20].

Serum antibodies to lipopolysaccharide (LPS)

LPS from *E. coli* O1, O2, O4, O14, O25, O26, O29, O55, O91, O103, O104, O105, O111, O113, O115, O118, O127, O128, O136, O145, O153, O157, O163 and O164 was prepared by digestion with proteinase K at 60°C for 1 h [21]. Sera were tested for antibodies to LPS by the line blot immunoassay technique [22]. Alkaline phosphatase-labelled antibodies against human IgM and IgA were used as detecting reagents, and enzymic immunodetection was performed with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium. Controls included O157-positive and -negative human serum samples, *Shigella dysenteriae* 1, *Salmonella enterica* serovar Urbana and *Yersinia enterocolitica* O9, with the last two antigens cross-reacting with O157 antigen.

RESULTS

Epidemiological and trace-back investigations

Following interviews with 73 guests, ten cases were identified with a date of onset of illness between 30 June and 3 July 2002. Nine cases were adults (age range 25–65 years), and one was a child aged 4 years. Besides diarrhoea, the most common symptoms reported were abdominal cramps (70%) and bloody diarrhoea (40%). Two adults developed HUS and were hospitalised. No deaths were reported.

Nine of the ten cases reported consumption of lightly roasted mutton (consumed pink by at least four individuals), and eight reported consumption of poultry pâté. Consumption of the roast mutton was not associated with illness (relative risk 1.8; 95% CI 0.2–12.9). Only consumption of poultry pâté tended to be associated with illness (relative risk 3.4; 95% CI 0.8–14.4). No other foods or drinks were associated with an increased risk of illness.

The trace-back investigation revealed that the two sheep were slaughtered at a local farm and cooked as spit-roasted mutton, and that the poultry pâté was produced industrially and distributed nationally. No poultry pâté specimens from the meal consumed on 29 June were available for testing. No additional cases of HUS related to consumption of poultry pâté were reported by hospital nephrology departments throughout France.

Molecular screening for Shiga toxin genes *stx1* and *stx2*

Among the stool specimens taken from five diarrhoea cases, two (40%) were positive for *stx* genes by PCR, indicating the presence of STEC. From these two samples, two different STEC strains were isolated. An *stx1*, *eae*, *ehxA*-positive isolate that gave a positive agglutination result for serogroup O26 was isolated from a case with diarrhoea, and an *stx2c*-positive isolate with an undetermined serogroup was obtained from one HUS case (Table 2).

Among the 24 food, environmental and animal samples analysed, 15 (63%) were positive for *stx* genes and none was positive for the *uid*A gene. Six STEC strains were isolated from the 15 *stx*-positive samples, following colony hybridisation: three from mutton and offal (liver and kidney), two from poultry pâté (two different batches) and one from sheep faeces.

Table 2. Characteristics of patient, food and animal STEC isolates, June–July 2002

Strain origin	Virulence characteristics	Serogroup[a]	Ribotype	PFGE pattern
HUS case	*stx2c*	R-148[b]	M2	P2
Diarrhoea case	*stx1*, *eae*, *ehxA*	R-26b[c]	M1	P1
Mutton	*stx2c*	R-148[b]	M2	P2
Sheep kidney	*stx2c*	R-148[b]	M2	P2
Sheep liver	*stx2c*	R-148[b]	M2	P2
Sheep faeces	*stx1*, *stx2*, *ehxA*	R-X	M4	P2
Poultry pâté (batch 1)	*stx2c*	R-Y	M3	P3
Poultry pâté (batch 2)	*stx2c*	R-Z	M4	P4

[a]R, PCR serogroup.
[b]Serotype O148:H8 confirmed by the International *Escherichia and Klebsiella* Centre (WHO) (Copenhagen).
[c]Serogoup O26.

The five isolates from food samples had the same toxin type (*stx2c*), but the isolate from sheep faeces had a different toxin type (*stx1*, *stx2*, *ehxA*) (Table 2). No isolates harboured the *eae* gene.

PCR amplification of the O-antigen gene cluster

For the seven food or stool strains of undetermined serogroup, PCR amplification of the O-antigen gene cluster identified five O-patterns (Table 2); the principal O-pattern was R-148, seen in four isolates from one HUS case, as well as in isolates from the mutton and offal samples. The other isolates had different O-patterns, referred to as R-X, R-Y and R-Z. The isolate with O-pattern R-148 was confirmed as *E. coli* O148:H8 by the International *Escherichia* and *Klebsiella* Centre.

Ribotyping and PFGE of STEC isolates from patients and food

During this outbreak, eight STEC isolates were examined by ribotyping and PFGE. Four distinct ribotypes and four distinct PFGE patterns were observed among the eight STEC isolates (Table 2). Ribotype M2 was seen in four (50%) isolates, and ribotypes M4, M1 and M3 were seen in two (25%), one (13%) and one (13%) isolate, respectively. PFGE pattern P2 was seen in five (63%) isolates, and patterns P1, P3 and P4 were seen in one (13%) isolate each.

Serum antibodies to LPS

No antibodies against the O antigens included in the panel tested were detected in the sera of the two HUS cases. O148 was not included in the panel.

DISCUSSION

In order to identify the origin of this outbreak, it was necessary to collect the complete epidemiological, microbiological and veterinary data. All these data suggested that the outbreak was caused by *E. coli* O148:H8 infection, linked to the consumption of undercooked mutton. Mutton was consumed by 90% of the patients, including the two HUS cases, and the microbiological investigation revealed that the three STEC isolates from the mutton and the offal were indistinguishable from the isolate from one of the HUS cases (*stx2c* toxin type, P2 PFGE pattern, M2 ribotype and O148 serogroup), whereas the pâté isolates differed.

The investigation emphasised the need to combine epidemiological, microbiological and environmental investigations. Thus, although the results of the cohort study were unable to pinpoint the mutton as a source of the infection, this could be explained by the fact that the two sheep carcasses, cooked whole on the spit-roast, were probably contaminated initially in a heterogeneous manner (i.e., accidental contamination with intestinal contents at slaughter time), and then secondarily according to the variable degrees of cooking resulting from the positions of individual pieces of meat (i.e., from the surface of the carcass or from deep down). These hypotheses were not confirmed, as the precise pieces of meat consumed were not identifiable by the guests. No association was found between eating pink (undercooked) meat and illness. However, infection caused by consumption of contaminated mutton is plausible. Outbreaks of *E. coli* O157:H7 infection associated with contact with sheep or consumption of mutton have been described previously [23–25]. Like all ruminants, sheep are reservoirs of STEC [26–29], and prevalence studies in slaughterhouses have shown that meat from sheep (lamb sausages, minced mutton, sheep carcasses) can be contaminated by STEC [24,30].

During these investigations, four different strains (different molecular serogroups, distinct ribotypes and distinct PFGE patterns) were isolated from food of various origin. Isolation of an STEC strain from a food sample does not permit a conclusion that the food is the origin of an outbreak or of a sporadic case [28,31]. In particular, the presence of *stx* genes in a food sample simply indicates possible contamination and not necessarily the presence of viable STEC in the sample. In the present investigation, it would not have been possible to identify the origin of the contamination without using complementary discriminatory techniques to characterise the STEC isolates (serogrouping, ribotyping and PFGE).

The STEC isolates were recovered from two of the five patients from whom stool samples were obtained. However, a negative STEC result in a stool sample obtained 10–11 days after onset of diarrhoea does not exclude a diagnosis of STEC infection. The excretion of STEC in stools is short-term, and often for <10 days following exposure [32]. Moreover, serology, regarded usually as the most efficient method for detection at 1 week following diarrhoea, could not be used to confirm the STEC infection because the LPS of the implicated serotype was not included in the test panel. These results illustrate the limitations of both stool culture and serology when used as single diagnostic tools, and emphasise the need to combine these two methods with clinical criteria in the diagnosis of STEC infections. The isolation of two different STEC strains (O148 and O26) from the patients could be explained, in an outbreak context, either by infection of the patients following consumption of meat that was itself contaminated by several strains, only one of which was isolated from the meat, or by concomitant infection of one of the patients [33] by another strain not implicated in this outbreak.

E. coli O148 belongs to the group of enterotoxigenic *E. coli*, and has been described previously as being responsible for diarrhoea in children and adults in several countries [34–38]. An *E. coli* O148:H8 strain (*stx2d*-positive and *eae*-negative) was isolated from a case of bloody diarrhoea in Germany [39]. In the present outbreak, the *E. coli* O148 strain isolated was associated with haemorrhagic colitis and HUS, and possessed the *stx2c* gene, which confirms its characterisation as an STEC strain. *E. coli* O157:H7 is found most frequently during sporadic infections or outbreaks, but other non-O157 serogroups (O26, O103, O111, O121, O145, O153, etc.) have also been implicated in STEC infections and HUS [33,40,41]. However, the actual frequency of non-O157 serogroups is difficult to estimate, and is probably underestimated because of unsuitable detection methods or a failure to search for non-0157 serogroups [1].

The strain implicated in the present outbreak possessed only the *stx2c* gene. The absence of the

eae and *ehxA* genes does not appear to have diminished its virulence, but other pathogenicity traits (e.g., *saa*, *cnf*, *katP*) were not studied. Clinical symptoms observed in STEC infections are associated primarily with the production of *stx* [42], and *stx2* and s*tx2c* have both been associated with increased virulence of STEC [43,44]. The *eae* gene, when present in STEC, produces intimin, which is associated significantly with bloody diarrhoea and HUS [45], but may not be essential for the development of HUS in adults [46]. Enterohaemolysin, which has been suspected of playing a role in pathogenicity following the finding of anti-EhxA antibodies in patients [14], is not present in all STEC isolates from clinical samples, and its impact on pathogenicity is still a matter for debate. Thus, the role of virulence factors found in classical microbiological investigations of STEC infections should be interpreted according to the clinical and epidemiological context. Patients with HUS in the present outbreak were adults, who are regarded as being less susceptible to developing HUS after STEC infection. This suggests that the pathogenesis of STEC in adults has not yet been fully elucidated, and that additional virulence factors in these strains may remain to be identified.

Genes coding for virulence factors were found in food products that were both associated and non-associated with the occurrence of cases. This illustrates the difficulty of determining whether a strain of STEC isolated from a food or animal sample is pathogenic or not, and also shows that the conclusion cannot be based exclusively on the presence of genes coding for virulence factors, or on the number of different genes found. The results described in this study also demonstrate that molecular subtyping is an essential complement to epidemiological investigations in order to identify the source of an outbreak of non-O157 STEC infections.

ACKNOWLEDGEMENTS

The authors would like to thank the medical staff of the Libourne Hospital, the local public health authorities of Gironde and the local veterinary services of Gironde and Sarthe, who assisted with this investigation.

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