Acta vet. scand. 2000, *41,* 167-171.

Listeria monocytogenes in Faeces from Clinically Healthy Dairy Cows in Sweden

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Unnerstad H, Romell A, Ericsson H, Danielsson-Tham M-L, Tham W: *Listeria monocytogenes* in faeces from clinically healthy dairy cows in Sweden. Acta vet. scand. 2000, 41, 167-171. – Faecal samples from 102 clinically healthy dairy cows, representing 34 farms in the Swedish province of Uppsala, were analysed for the presence of Listeria spp. using an enrichment procedure. *Listeria monocytogenes* was isolated from six (6%) and *L. innocua* from 2 (2%) cows. From each of the 6 samples positive for *L. monocytogenes,* 5 isolates were further characterised by restriction enzyme analysis using the 3 enzymes *Apa* I, *Sma* I, and *Asc* I, followed by pulsed-field gel electrophoresis. Three of the *L. monocytogenes* positive cows lived at the same farm, and they all harboured the same clonal type. One of these 3 cows also harboured a further clonal type of *L. monocytogenes.* The fact that one of the cows harboured 2 different clonal types of *L. monocytogenes* is important from an epidemiological point of view when routes of infection are to be investigated.

Listeria monocytogenes; cow; faecal samples; frequency of carriers; clonal type; restriction enzyme; pulsed-field gel electrophoresis.

Introduction

Listeria monocytogenes is a Gram positive rod that can infect both humans and animals with severe consequenses such as abortion, septicaemia, and involvement of the CNS (*Seeliger & Jones* 1986). The pathogen may be present in a wide range of food (*Farber & Peterkin* 1991) as well as in certain forage products (*Hird & Genigeorgis* 1990), some of which are considered important sources of listeriosis. Animals and humans may also be clinically healthy carriers of the bacterium (*Husu* 1990, *Schönberg & Gerigk* 1991).

The aim of this study was to investigate the frequency of clinically healthy carriers of *L. monocytogenes* among dairy cows in the province of Uppsala, Sweden, and to characterise the isolates obtained by use of restriction enzyme analysis (REA) with PFGE.

Materials and methods

Faecal samples

This study included 34 randomly selected farms in the province of Uppsala, Sweden. Three clinically healthy cows were sampled on each farm, for a total of 102 cows. From each cow, a handful of faeces was collected directly from the rectum, by use of a plastic rectal glove, and put into a sterile plastic vial. The samples were kept at 4 °C until analysis.

Listeria isolation and confirmation

The *Listeria* analyses were carried out mainly according to the procedure described by the International Dairy Federation (1990). The method can briefly be described as follows: 25 g faeces from each sample was transfered into 225 ml Listeria Enrichment Broth (Oxoid, Basingstoke, Hampshire, England and Difco, De-

Table 1. REA profiles of 30 *L. monocytogenes* strains isolated from 6 cows.

Strain designation	Cow	Farm	Restriction enzyme profiles			Clonal type
			Apa I	Sma I	Asc I	
SLU 408-412	1	1	A	B	C:1	I A
SLU 413-417	2	1	A	B	C:1	I A
SLU 418-421	3	1	A	B	C:1	I A
SLU 422	3	1	D	E	F	II
SLU 423-427	4	2	G	H	I	III
SLU 428-432	5	3	A	B	C:2	I B
SLU 433-437	6	4	A	B	J	IV

troit, MI, USA) supplemented with acriflavine, nalidixic acid, and cycloheximide in accordance with the IDF Standard 143:1990. The broth was incubated at 30 °C for 48 h. The pH of the incubated enrichment broth was measured with a pH meter after 48 h (Orion Research model 701, Cambridge, MA, USA) to ensure that the pH was not too low for *L. monocytogenes* growth. Then, 0.1 ml of the enrichment broth was spread with a glass spatula onto an Oxford agar plate (Oxoid). The agar plates were incubated at 37 °C and examined after 24 h and 48 h. When typical *Listeria* colonies had grown, 5 colonies from each plate were isolated, subcultured on horse blood agar, and confirmed. Bacterial cells were tested for Gram reaction, cell shape, haemolytic reaction on horse blood agar, tumbling motility at 20 °C, fermentation of mannitol, ramnose and xylose, production of catalase, and hydrolysis of esculine (*Seeliger & Jones* 1986). All *L. monocytogenes* isolates were serotyped with *Listeria* O Antiserum type 1 and 4 (Difco Laboratories, Detroit, MI, USA). Two *L. monocytogenes* isolates out of the 5 from each positive cow (including strain 422, Table 1) were further serotyped according to reference method (*Seeliger & Höhne* 1979). All *L. monocytogenes* isolates were frozen in 80% Brain Heart Infusion Broth (Merck, Darmstadt, Germany) and 20% glycerol, vol/vol, –70 °C, for further, later, characterisation.

Preparation of gel plugs for PFGE

The procedure described is based on the method of *Brosch et al.* (1991) and the Pharmacia LKB Biotechnology protocol for preparing *Escherichia coli* DNA (*Pharmacia*). One loopful of each freeze-stored bacterial culture was streaked onto horse blood agar and incubated at 37 °C for 24 h. One well-isolated colony was inoculated into 10 ml Brain Heart Infusion Broth (Difco) in a centrifuge tube and incubated at 37 °C for 18-24 h. The tube was then centrifuged (10 min, 310 g, 4 °C); the pellet was washed once with 10 ml Pett IV (10 mM Tris HCl, pH 7.6, 1 M NaCl), centrifuged again, and then resuspended in 2.4 ml Pett IV. Of the bacterial suspension, 500 µl was heated to 40 °C and mixed with 500 µl of 1% low-melt agarose solution (InCert Agarose, FMC BioProducts, Rockland, ME, USA) of the same temperature. The mixture was distributed into insert moulds (110 µl in each mould) and then cooled for 30 min at 4 °C. The gel plugs were incubated overnight at 37 °C in a solution of 3 mg lysozyme (Boehringer Mannheim GmbH, Mannheim, Germany) per ml 0.1 M phosphate buffer (pH 7.0, with 20% sucrose); washed 5 min in TE-buffer and incubated in 0.5 M EDTA, 1% N-lauroyl-sarcosine (Sigma, St Louis, MO, USA) with 2 mg/ml Pronase E (Boehringer Mannheim); and incubated at 55 °C for 48 h with the solution changed after 24 h. The plugs were sliced in longitudinal halves (about 50 µl each)

and washed in TE-buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Pronase E was deactivated with 350 μg Pefabloc® SC (Boehringer Mannheim) per ml TE-buffer for 2 h at 50 °C and, finally, the plugs were washed again in TE-buffer.

Restriction enzyme cleavage and PFGE

The restriction enzymes *Apa* I, *Sma* I (10 u/μl, Boehringer Mannheim) and *Asc* I (10 u/μl, New England Biolabs, Beverly, MA, USA) were used as recommended by the manufacturers. For *Apa* I and *Sma* I, half a gel plug (10^8 bacteria) was incubated overnight at 30 °C and 25 °C, respectively, in 25 u enzyme, 11.5 μg acetylated BSA (bovine serum albumin; Promega, Madison, WI, USA), 11.5 μl Buffer A (incubation buffer for restriction enzymes, 10 × conc., Boehringer Mannheim), and sterile water up to 115 μl. For *Asc* I half a gel plug was incubated overnight in 14 u enzyme, 11.5 μg acetylated BSA, 12.6 μl NE Buffer 4 (10 × conc., New England Biolabs), and sterile water up to 115 μl at 37 °C. Plugs with *Escherichia coli* (G5244) cleaved with *Xba* I were used as markers. The plugs were cast in a 1.4% Fastlane Agarose gel (FMC BioProducts, Rockland, ME, USA) and run in a Pharmacia Gene Navigator (Pharmacia, Uppsala, Sweden) at 9 °C with circulating 0.5 × TBE-buffer (45 mM Tris-borate, 1 mM EDTA). The running programme consisted of continously interpolated pulses from 4 to 40 s during 24 h under a voltage of 200 V. The gel was stained in ethidium bromide solution (final concentration 0.75 μg/ml) for 20 min, washed for 20 min in 0.5 × TBE-buffer and photographed over a 312 nm transilluminator. The lanes on the gel were visually examined. DNA was prepared twice from each strain and analysed by PFGE to ensure repeatability.

Results

The pH of the enrichment broth after 48 h incubation averaged 6.0 (range: 5.6-6.3). *Listeria* spp. were isolated from faecal samples from 8 out of the 102 (8%) clinically healthy dairy cows. Six of the cows harboured *L. monocytogenes* (6%) and 2 cows *L. innocua* (2%); the *L. innocua* strains were not further investigated. Three of the *L. monocytogenes* positive cows lived at the same farm (farm 1); the others were located at different farms. Altogether 6 cows rendered 30 (6×5) isolates of *L. monocytogenes*. All the isolated *L. monocytogenes* strains belonged to serogroup 1/2. Two strains from each positive cow (altogether 12 strains) were further serotyped according to reference method (*Seeliger & Höhne* 1979), and all these strains belonged to serovar 1/2a. The 30 isolated *L. monocytogenes* strains were characterised by pulsed-field gel electrophoresis (Fig. 1,

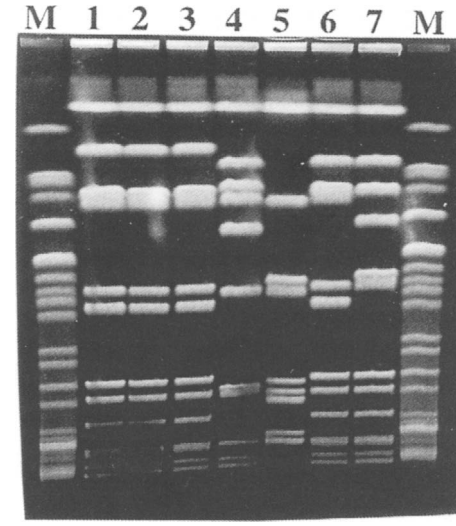

Figure 1. REA profiles of *L. monocytogenes* strains from faeces of clinically healthy dairy cattle. The DNA is cleaved with *Asc* I. M, *E. coli* (G5244) cleaved with *Xba* I used as marker; lane 1, strain SLU 409 profile C:1; lane 2, strain SLU 414 profile C:1; lane 3, strain SLU 418 profile C:1; lane 4, strain SLU 422 profile F; lane 5, strain SLU 424 profile I; lane 6, strain SLU 429 profile C:2; lane 7, strain SLU 434 profile J; M, *E. coli* (G5244) cleaved with *Xba* I.

Table 1). The strains could be grouped into 5 clonal types according to their restriction enzyme profiles. The 3 *L. monocytogenes* positive cows from the same farm (cows no. 1, 2, and 3) harboured strains with indistinguishable restriction profiles with all 3 enzymes (clonal type I A). In addition, cow no. 3 also harboured another clonal type of *L. monocytogenes* with clearly different profiles with all 3 enzymes (clonal type II). The isolates from the other 3 cows from farms 2, 3, and 4 showed difference in restriction profile with at least one enzyme (clonal types III, I B, and IV).

The results of the second DNA preparation and PFGE analysis were identical with the results of the first.

Discussion

The isolation of *L. monocytogenes* from faeces from 6% of the dairy cows is well in line with the results of Husu (1990) who isolated *L. monocytogenes* from faeces from 6.7% of 3,878 randomly selected dairy cows representing 249 farms in Finland during 2 years. The shedding of *L. monocytogenes* from clinically healthy cows shows that cattle faeces could be a source of contamination of milk and carcasses with *L. monocytogenes,* and thus may contribute to foodborne listeriosis. Other studies have shown that food products can be contaminated with *L. monocytogenes* in the processing industry (*Boerlin & Pifaretti* 1991, *Rørvik et al.* 1995, *Unnerstad et al.* 1996, *Ericsson et al.* 1997).

The 3 *L. monocytogenes* positive cows that lived at the same farm all harboured clonal type I A of *L. monocytogenes.* This is not surprising because these cows lived in the same environment and consumed the same forage. However, one of the 3 cows (no. 3), also harboured another clonal type, no. II. The profiles of that strain (SLU 422) were completely different with all 3 enzymes and may be considered unrelated to clonal type I A. A human faecal carrier excreting 2 different serovars of *L. monocytogenes* has been reported (*MacGowan et al.* 1991). More than one clonal type of *L. monocytogenes* have also been found in brain tissue of a fallow deer (*Tham et al.* 1999) as well as in a soft cheese (*Danielsson-Tham et al.* 1993). The fact that there may be more than one clonal type of *L. monocytogenes* present in the same sample is essential. In a case, or outbreak, of listeriosis, it is urgent to trace the origin of infection in order to inhibit further spreading. The conceivable presence of several clonal types makes it interesting to characterise several isolates from both the patients and the suspected food or forage items in order to maximize the possibility of establishing an epidemiological link.

In the present study, clonal type I B had restriction profiles that were indistinguishable from clonal type I A with 2 enzymes, but differed with a single genetic event (*Tenover et al.* 1995) with the enzyme *Asc* I (Table 1). Hence, the isolates from cow no. 5 may be considered closely related to clonal type I A, based on the restriction profiles. However, since a link is not firmly established, the epidemiological relationship is uncertain.

Acknowledgements

This study was supported financially by Stadsveterinär Billströms Foundation, to which we express our gratitude. The Department of Ambulant Service, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences is gratefully acknowledged for collecting the faecal samples, and Prof. J. Bille and Dr. E. Bannerman, Medical Bacteriology Laboratory, University Hospital, Lausanne, Switzerland for serotyping 12 of the strains.

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Sammanfattning

Listeria monocytogenes i träckprover från kliniskt friska mjölkkor

Träckprover från 102 kliniskt friska kor i Uppsala kommun analyserades med avseende på förekomst av *Listeria* spp. *Listeria monocytogenes* isolerades från sex och *L. innocua* från 2 kor. Från varje *L. monocytogenes*-positivt prov karakteriserades 5 isolat genom restriktionsenzymklyvning med enzymerna *Apa* I, *Sma* I och *Asc* I följt av pulsfältsgelelektrofores. Samma klontyp av *L. monocytogenes* isolerades från 3 kor från samma gård. Från en av dessa kor isolerades dessutom ytterligare en klontyp.

(Received November 16, 1999; accepted February 11, 2000).

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