Listeria monocytogenes translocates throughout the digestive tract in asymptomatic sheep

Etienne Zundel and Serge Bernard

INRA, Infectiologie Animale et Santé Publique, 37380 Nouzilly, France

Ruminants are fed forage which is often contaminated with Listeria, and frequently shed Listeria monocytogenes with their faeces. The present study was designed to localize the sites of infection in the digestive tract concomitant with Listeria faecal excretion in a sheep model. Ten Listeria-free sheep were inoculated per os with a dose of 10⁶ c.f.u. of a pathogenic L. monocytogenes strain. Listeria received by two of the ten animals were radiolabelled with ¹¹¹indium oxine. The dissemination of the Listeria was assessed by in vivo imaging, by culture of bacteria in the faeces, organs and digesta samples taken at slaughter on days 1, 2, 6, 10 and 14 post-inoculation, and by measuring gamma radioactivity of samples on day 6. It was shown that Listeria spread through the entire volume of the forestomachs within 4 h, and through the whole gastrointestinal tract (GIT) within 24 h. Faecal shedding of Listeria lasted 10 days. Rumen, duodenum, jejunum, ileum, caecum and colon walls and digesta, mesenteric lymph nodes, liver and spleen were temporarily infected. However, Listeria persisted for at least 14 days in rumen digesta and retropheangeal lymph nodes, and at a relatively high level (1 x 10⁴ c.f.u. g⁻¹) in palatine tonsils. These findings suggest that L. monocytogenes can translocate from all parts of the GIT, with the rumen and reticulo-rumen, but not the gallbladder, serving as a reservoir. The results indicate that brief and low-level faecal excretion of L. monocytogenes is concomitant with a transitory asymptomatic infection in sheep.

INTRODUCTION

The Gram-positive bacterium Listeria monocytogenes is widespread in nature and frequently contaminates fodder (Fenlon, 1999). It is associated with several diseases (listeriosis) in both humans and animals (Low & Donachie, 1997). Humans generally become infected with L. monocytogenes by consuming contaminated food products (Slutsker & Schuchat, 1999). Sheep, cattle and goats often shed L. monocytogenes in their faeces without symptoms (Wesley, 1999). The farm sources of contamination for human foods such as milk are the animals themselves and the environment (McLauchlin, 1997). Since L. monocytogenes mastitis is rare (Jensen et al., 1996), raw milk is mainly contaminated from the environment for human foods such as milk are the animals themselves and the environment (McLauchlin, 1997).

Listeria monocytogenes is a Gram-positive, non-spore-forming coccoidbacillus that is widely distributed in nature and frequently contaminates fodder (Fenlon, 1999). It is associated with several diseases (listeriosis) in both humans and animals (Low & Donachie, 1997). Humans generally become infected with L. monocytogenes by consuming contaminated food products (Slutsker & Schuchat, 1999). Sheep, cattle and goats often shed L. monocytogenes in their faeces without symptoms (Wesley, 1999). The farm sources of contamination for human foods such as milk are the animals themselves and the environment (McLauchlin, 1997). Since L. monocytogenes mastitis is rare (Jensen et al., 1996), raw milk is mainly contaminated from the environment. The present study was designed to localize the sites of infection in the digestive tract concomitant with Listeria faecal excretion in a sheep model. Radiolabelled or non-radiolabelled L. monocytogenes was orally administered to animals, and dissemination was assessed by in vivo imaging, bacteriological detection and gamma radioactivity measurements at slaughter. Our results indicate that brief and low-level faecal excretion of L. monocytogenes is concomitant with a transitory asymptomatic infection in sheep, after

Abbreviations: GIT, gastrointestinal tract; MPN, most probable number; PI, post-inoculation; ROI, region of interest.

Scintigraphic imaging of the spread of L. monocytogenes radiolabelled with ¹¹¹In oxine in sheep nos 5 and 6 is shown in Supplementary Fig. S1 with the online version of this paper.

Fig. S1 with the online version of this paper.
translocation from every segment of the gastrointestinal tract (GIT), with the rumen digesta serving as a reservoir.

METHODS

Animals. Ten Lacaune sheep were born and reared in a Listeria-free environment in category 3 containment level facilities of our laboratory. They were housed together on a duckboard, exposed to a light–dark cycle of 24 h (light–dark 12:12), and fed pelleted alfalfa and flattened cereals ad libitum. Sheep were 8 months old and weighed 53.3±3.8 kg at the time of oral inoculation with L. monocytogenes [day 0 (D0)]. The use of animals complied with the European Directive 86/609/EEC (1986/11/24).

L. monocytogenes inoculum and labelling with 111Indium oxine. The LCCN 95-962 L. monocytogenes strain serotype 1/2a had previously been isolated in our laboratory from a ewe with clinical listeriosis. In vivo and in vitro virulence tests (Roche et al., 2001) have confirmed the virulence observed in the field. We labelled bacteria with 111In oxine (half-life 67 h), because radioactivity is incorporated very rapidly (90% within 10 min) with little damage to bacteria, and 111In released from the cells would not be reutilized (Ardehali & Mohammad, 1993). In brief, a 24 h culture on tryptic soy agar (TSA; bioMérieux) was suspended, washed and spectrophotometrically titrated to 4×10^10 c.f.u. ml⁻¹ in 4.5 ml PBS. The bacterial suspension was centrifuged (4000 g, 4°C for 30 min). The supernatant was discarded and the pellet was mixed with 0.2 ml 74 mBq 111In oxine (Mallinckrodt). The solution was corrected to pH 6 with 0.2 M Tris. After 15 min at room temperature, the reaction was stopped with 20 ml PBS with 10% fetal calf serum (Invitrogen). The labelled bacteria were washed twice in PBS by centrifugation. We achieved a labelling efficiency of 64% (percentage of radioactive inoculum was not different from that of mock-labelled bacteria that had been processed under the same conditions, except for the absence of the radioactive oxine.

Cold and radiolabelled inocula were adjusted to 10¹¹ c.f.u. ml⁻¹. In order to mimic natural oral contamination, cotton swabs (bioMérieux) were impregnated with 100 µl inoculum (10¹¹ c.f.u. ml⁻¹) and fed to the two animals selected for in vivo imaging on D0, 2 h after normal feeding.

Scintigraphic imaging. We chose scintigraphy to monitor the spread of bacteria because it is a non-invasive technique usable in vivo. The sheep were anaesthetized with a 5–2% halothane in air in a light–dark cycle of 2:1 min⁻¹. On D0 [30 min and 4 h post-inoculation (PI)], D1, D2, D3 and D6, ventral and lateral (right decubitus) 2–10 min static images were acquired using a gamma camera (Opti-5G) equipped with a high resolution, low-energy, parallel collimator. Data were recorded using a 15% window centred on the 170 keV photopeak of 111In into a 256×256 pixel matrix on a computer system specialized in digital display and analysis (Mirage, Segami). Distribution of radioactive bacteria was assessed from static images using regions of interest (ROI) for different body areas (head, thorax, abdomen and pelvis). After correction for radioactive decay, the count rate recorded within these regions was used to calculate the percentage of radioactivity injected on D0 for each ROI, and to edit images (available as Supplementary Fig. S1 in JMM Online).

Sample radioactivity at slaughter. Sheep nos 5 and 6 were slaughtered on D6, and samples were collected as described below. Immediately before culturing for Listeria, 1 ml of each sample suspension in half-Fraser broth was counted (Cobra gamma counter, Packard). The radioactivity was expressed as disintegrations per minute per gram of sample (d.p.m. g⁻¹). The residual radioactivity in organs at slaughter indicated the presence of live or formerly live bacteria. By counting the radionuclide and bacterial counts in serial dilutions of the labelled suspension, it was determined that a count of 3 d.p.m. corresponded to approximately one viable bacterium. The lower limit of detection of the gamma-counting system was 50–100 bacteria, depending on the background radioactivity level. Survival of bacteria was defined as the ratio of (numbers of Listeria counted on plates)/(numbers of Listeria calculated from d.p.m. counts) (Eaves-Pyles & Alexander, 2001). A ratio below one suggested that the overall number of bacteria decreased after translocation, and above one that it increased.

Sample collection. Sheep were subsequently monitored for symptoms, and their faeces, feed and drinking water were checked for Listeria once a month for 6 months after inoculation. Faeces and saliva were regularly taken in the morning before feeding, from live animals after inoculation (Table 1). Faeces were collected directly from the rectum, and placed in individual screw-capped specimen bottles, with gloves changed before each individual collection. Saliva samples (125 µl maximum volume) were taken with a plastic-rod rayon swab (Coplan). Samples were processed in our laboratory within 30 min of collection. Two sheep per day were euthanized with sodium thiopental on D1, D2, D6, D10 and D14 PI. Samples (up to 25 g when possible) were sequentially collected from 25 organs and digesta from all ten sheep. GIT segment samples included the oesophagus, rumen, duodenum, jejunum, ileum, caecum and colon (distant loop). A digesta sample was collected from each GIT segment, except the oesophagus. GIT tissue from a 20 cm (ileum) to 60 cm (jejunum) sample was rinsed with saline to remove visible digesta. Palatine tonsils, retropharyngeal and mesenteric lymph nodes as local lymphoid organs, and liver, spleen and blood [about 20 ml in evacuated blood-collecting tubes with lithium heparin (Venoject; Terumo)] as systemic organs were harvested.

Table 1. Number of animals with L. monocytogenes recovered before slaughter in ten sheep inoculated per os with 10¹⁰ c.f.u. L. monocytogenes on D0

<table>
<thead>
<tr>
<th>Days post-inoculation</th>
<th>No. of animals</th>
<th>Tested*</th>
<th>Positive</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Saliva†</td>
<td>Faeces‡</td>
</tr>
<tr>
<td>D1</td>
<td>10</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>D2</td>
<td>8</td>
<td>5</td>
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<td>D3</td>
<td>6</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>D6</td>
<td>6</td>
<td>4</td>
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</tr>
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<td>D7</td>
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<td>4</td>
<td>1</td>
</tr>
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<td>D8</td>
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<td>3</td>
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</tr>
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<td>D9</td>
<td>4</td>
<td>2</td>
<td>4</td>
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<td>D10</td>
<td>4</td>
<td>2</td>
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</tr>
<tr>
<td>D13</td>
<td>2</td>
<td>1</td>
<td>0</td>
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<tr>
<td>D14</td>
<td>2</td>
<td>0</td>
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</table>

*Two animals were slaughtered on D1, D2, D6, D10 and D14. †Maximal count, 3.6×10³ c.f.u. L. monocytogenes g⁻¹ on D2. ‡L. monocytogenes was isolated from most saliva samples only after the enrichment procedure.
**Listeria culture of samples.** Samples were tested using a bacteriologically selective enrichment test (AFNOR, 1997). In brief, a sample was incubated (24 h, 30 °C) in nine volumes of enrichment broth (half-Fraser, Oxoid). A 100 µl aliquot was then placed in 10 ml Fraser broth and incubated (24 and 48 h, 37 °C). This subculture (0-2 ml) was plated on Oxford agar (Oxoid) and incubated (24–48 h, 37 °C). *Listeria*-like colonies were confirmed as described below. This enrichment resulted in a positive or negative culture.

*Listeria* from rumen digesta samples were counted using the most probable number (MPN) method (Blodgett, 2001) in Fraser broth tubes. *Listeria* from saliva were counted with a direct plating method on TSA, and those from other samples were serially diluted and plated on Oxford plates (AFNOR, 1997). Quantitative results were expressed in c.f.u. g⁻¹. When necessary, *Listeria*-like colonies were streaked on TSA plates and confirmed by examining the colonies under obliquely reflected light (McClain & Lee, 1988), and by standard methods: catalase reaction (positive), haemolysis of horse blood agar (bioMérieux), Christie–Atkins–Munch–Petersen (CAMP) test, and acid production from D-xylose (negative), L-rhamnose (positive) and mannitol (negative). The results were confirmed with API Listeria (bioMérieux) when necessary.

Because of dilution factors, the lowest detectable counts were 1 c.f.u. per 25 g (MPN), 50 c.f.u. per g or ml (direct plating) and 200 c.f.u. per ml (direct plating with saliva). The limit of detection of the enrichment technique was 1 c.f.u. per 25 g. Therefore samples positive after enrichment contained between 0.04 and 50 or 200 c.f.u. g⁻¹. To facilitate reading of graphical representations, we arbitrarily assumed enrichment results to correspond to 10 c.f.u. g⁻¹ (Fig. 1).

**RESULTS AND DISCUSSION**

Sheep are naturally susceptible to listerial infection (Low & Donachie, 1997), whereas mice are not (Lecuit *et al.*, 2001; Marco *et al.*, 1997). In addition, the murine GIT is not representative of that of ruminants. Some studies in mice have focused on the digestive phase of listeriosis (Gahan & Hill, 2005) and have established that the gallbladder is colonized by *L. monocytogenes* (Hardy *et al.*, 2004), but no data are available for sheep. We chose a sheep model (Lhopital *et al.*, 1993) in order to assess the dissemination of orally administered bacteria in the body and to localize the site of infection in asymptomatic carriers with low-level faecal excretion of *L. monocytogenes*.

**Listeria excretion**

*Listeria* were not recovered from the faeces, feed or drinking water prior to inoculation. The experimental inoculation resulted in a low-level excretion in saliva for 13 days, and in

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**Fig. 1.** Mean numbers of *L. monocytogenes* in samples collected at slaughter (two sheep per day) of ten sheep inoculated *per os* with 10¹⁰ c.f.u. *L. monocytogenes* at D0. Abbreviations: R + L, right and left; retro., retropharyngeal; LN, lymph nodes.
faeces for a shorter time: up to 10 days (Table 1), in line with previous results (Lhopital et al., 1993). In another study, a $10^4$ or $10^6$ c.f.u. dose in sheep appeared to have no effect on faecal excretion (Zundel et al., 2006). All ten animals remained well, without symptoms, throughout the study, even when they shed *Listeria*, and thus were considered as asymptomatic carriers.

**Spread of Listeria**

After inoculation, scintigraphic images taken successively from the ROI were grouped in one figure (Supplementary Fig. S1). Body outline was added to the images (white line). The head and neck showed very little labelling 30 min or 4 h post-inoculation in sheep no. 5. The white spot in its rumen (arrows, Supplementary Fig. S1) corresponds to the swab head containing the inoculum, swallowed without chewing, as is usual for ruminants. It was seen again on D1 in the right subcostal area, and on D2 in the lower left side of the belly. The mouth of sheep no. 6 was labelled immediately after inoculation. Its inoculum swab having been chewed, *Listeria* spread more rapidly and more efficiently in its forestomachs during the first 4 h. In comparison, *Escherichia coli* O157 : H7 was isolated from the duodenum digesta 1 h after $2 \times 10^{11}$ c.f.u. *E. coli* was directly inoculated into the rumen of steers with cannulae inserted into both the rumen and duodenum (Grauke et al., 2002). But precise tomographic localization could only have been obtained by single photon emission computer tomography (SPECT), and was not possible using planar scintigraphic acquisition, although the camera used in this study facilitated quantitative analysis. The spread of *L. monocytogenes* in the lumen of the GIT, as seen with scintigraphy, is concomitant with bacterial translocation throughout the different segments of the GIT, as observed by culturing samples collected within 48 h of inoculation (Fig. 1). It is worthy of note that detectable *Listeria* dissemination was maximal and the rectum was labelled on D1 for both sheep (dashed lines, Supplementary Fig. S1).

**Listeria translocation to deep organs**

Bacteriological analyses at slaughter confirmed the presence of *Listeria* in all samples of two sheep within 24 h of inoculation, with the exception of blood and bile. The mean numbers of *Listeria* in samples are shown in Fig. 1 for digesta, GIT walls and lymphoid organs. The greatest number of *Listeria* in the GIT was approximately $10^7$ c.f.u. per gram of digesta on D1 and of walls on D2. Very few *Listeria* were isolated from digesta and walls in the distal part of the GIT. *Listeria* were recovered from mesenteric lymph nodes from D1 onwards, with a maximum number on D2 (Fig. 1). Numbers in the tonsils and the retropharyngeal lymph nodes were higher than those of mesenteric lymph nodes, probably because the doses were given on a swab and not with a worming gun. A few bacteria reached the liver and spleen ($<50$ c.f.u. g$^{-1}$), as systemic lymphoid organs. Thus our study revealed the ability of *Listeria* to translocate from every segment of the GIT.

Samples collected at slaughter (D6) from sheep nos 5 and 6 were measured for gamma radioactivity and bacterial enumeration (Fig. 2). Samples from sheep no. 6 were more often contaminated or infected with *Listeria* than those of sheep no. 5, although numbers were close to the lower detection limit. There was no real difference between the *Listeria* counts in the lymphoid organs of the head, although sheep no. 5 did not chew the inoculum swab. Twelve samples (saliva, blood, coeliac, jejunal, ileo-caecal and colic lymph nodes, oesophagus, rumen, duodenum, jejunum, ileum and colon tissues) had very low radioactivity, close to the background level, in these two sheep. Therefore, these samples were considered ‘double-negative’. The scatter diagram (not shown) of the radioactive samples from faeces, GIT digesta, caecum wall, palatine tonsils, retropharyngeal lymph nodes, spleen, liver and bile showed that both sheep dealt with *L. monocytogenes* in the same way ($R^2=0.932$).

In digesta samples collected at D6, an increase in radioactivity level was apparent downstream from the duodenum to the faeces, conversely to bacterial counts, but did not reach statistical significance ($P=0.075$, Kendall’s $W$ coefficient of concordance). The faeces samples were the most radioactive, with a *Listeria* survival ratio of almost 0. $	ext{In}$ is not excreted from bacteria, except when bacterial lysis occurs (Eaves-Pyles & Alexander, 1998). Moreover, $	ext{In}$ does not accumulate in the genitourinary and gastrointestinal tracts (Hughes, 2003). Therefore, *Listeria* elimination prevailed on proliferation in the GIT lumen. The low radionuclide counts of GIT walls and mesenteric lymph nodes suggest that only a part of the initial inoculum translocated and reached draining lymph nodes, where bacteria proliferate temporarily (D2, Fig. 1). Radiolabelled bacteria differentiated the role of the wall permeability to bacteria of the GIT lumen from that of the immune clearance of bacteria (Gianotti et al., 1995). The low to moderate radioactivity on D6 in the liver and spleen confirmed that *Listeria* cultured on D1 and D2 (Fig. 1) were eliminated. Therefore, our results show that the efficiency of *Listeria* translocation was low to moderate in sheep. The efficiency of listerial penetration is lower in mice (Marco et al., 1992) and in a rat ligated-ileal-loop model (Pron et al., 1998). The main point is that GIT lymph nodes were initially sites of temporary multiplication and then became sites of lysis and elimination of *Listeria*, in the same way as the liver, spleen and intestinal lumen.

**Listeria systemic infection**

The presence of *L. monocytogenes* in lymphoid organs, including spleen and liver, confirmed the systemic though subclinical course of infection after oral inoculation. An important finding was that no *Listeria* were isolated from bile, although sheep bile experimentally inoculated ($2 \times 10^7$ c.f.u. ml$^{-1}$) and incubated (24 h, 37°C) allowed *L. monocytogenes* LCCN 95-962 to grow up to $2 \times 10^8$ c.f.u. ml$^{-1}$. The same growth was observed in C57/B16 mice bile. Therefore it does not seem that excretion with bile would cause faecal excretion of *Listeria* in sheep, as
shown in mice with a sublethal dose (Briones et al., 1992; Marco et al., 1997), or an enterohepatic circulation of \textit{L. monocytogenes} (Hardy et al., 2004). Radioactivity in tonsils and retropharyngeal lymph nodes was moderate, but \textit{Listeria} counts remained high for 2 weeks, thus suggesting that bacteria increased in number in these lymphoid organs, which can therefore be considered as sites of multiplication (Fig. 2). This result is strengthened by the particularly high survival ratio of \textit{L. monocytogenes} (over 400) in tonsils. Tonsils provide good evidence of food-borne contamination, as shown in pigs, in which 22% of tonsils have been demonstrated to be \textit{Listeria} positive in different slaughterhouses (Autio et al., 2004). Tonsils can also be used by bacteria such as \textit{Salmonella} Typhimurium as a site of chronic infection up to 140 days after inoculation, resulting in asymptomatic carrier animals (Horter et al., 2003). However, under our conditions, the sheep tonsils were not able to contribute significantly to \textit{Listeria} faecal excretion, since they were situated upstream of the GIT, and they remained infected at up to a total count of $5 \times 10^7$ c.f.u. after the faecal excretion of \textit{Listeria} had stopped.

**Persistence of \textit{Listeria} infection**

\textit{Listeria} numbers in tonsils and retropharyngeal lymph nodes were stable until D14, probably due to local growth of bacteria and rumination. Ruminants regurgitate foodstuff from the rumen, and chew it again for 8 h per day. In fact, \textit{Listeria} continued to be isolated from rumen digesta and the duodenum wall on D14. There were more than $1 \times 10^5$ \textit{Listeria} g$^{-1}$ in rumen digesta (volume $\sim 10$ l) on D1, decreasing to 15 c.f.u. g$^{-1}$ on D14. Radioactivity in rumen digesta was moderate, with $10^5$ c.f.u. ml$^{-1}$ at D6, i.e. at least 10$^7$ c.f.u. \textit{Listeria} in the total content, with a survival ratio of 9. The renewal of the rumen content is continuous, and takes on average about 20 h for dry matter (Cannas et al., 2003). Therefore, \textit{Listeria} probably multiplied to make up in part for the permanent through-flow, the negative effects of interaction with rumen flora (around $10^6$ ciliate protozoa, $10^8$–$10^{11}$ bacteria and $10^8$ bacteriophages per millilitre), and even anti-\textit{Listeria} bacteriocins (Laukova & Czikkova, 1998). Conversely, \textit{E. coli} O157 : H7 does not seem to persist in the sheep rumen (Grauke et al., 2002). Therefore, a remarkable finding in our study was that the rumen content, like tonsils and retropharyngeal lymph nodes, was a long-lasting site of \textit{Listeria} multiplication. The rumen can thus be considered as a reservoir for \textit{L. monocytogenes}, discharging \textit{Listeria} downstream into the GIT for at least 2 weeks after a single per os inoculation. However, faecal excretion of \textit{Listeria} stopped before the bacterium disappeared from the head lymphoid organs, the rumen content and the duodenum wall (the enrichment limit of detection was
In conclusion, our results strongly suggest that the sporadic, intermittent and short-term faecal excretion of *L. monocytogenes* in ruminants given contaminated food on the farm is not a simple passage of the bacteria, but is associated with a transitory multiplication in the rumen content and a transitory infection, i.e. an asymptomatic carrier state.

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