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Short communication

Repeated daily doses do not increase *Listeria monocytogenes* infection in ewes as shown by faecal excretion and serological monitoring

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Abstract

Ruminants fed contaminated forage may shed *Listeria monocytogenes* in their faeces, and prolonged low daily doses of *L. monocytogenes* could cause listerial infection [Maijala, R., Lyytikainen, O., Autio, T., Aalto, T., Haavisto, L., Honkanen-Buzalski, T., 2001. Exposure of *Listeria monocytogenes* within an epidemic caused by butter in Finland. Int. J. Food Microbiol. 70, 97–109]. To compare listerial infection following single or repeated doses and the contamination of the environment with the excreted bacteria, ewes were orally inoculated with either 10^4, 10^6 or 10^{10} cfu *L. monocytogenes* once, or daily for 10 days. Serological responses were monitored with indirect ELISAs using recombinant listeriolysin O (LLO), internalin A (InlA) and internalin A-related protein (IrpA). The 24 inoculated animals displayed no symptoms, except for a transient hyperthermia in two animals given 10^{10} cfu. One ewe died on day 9 after non-listerial mastitis followed by listerial septicaemia. *L. monocytogenes* was recovered from day 1 post-inoculation until day 17 from the faeces of ewes inoculated with 10^6 or 10^{10} cfu. No antibodies were detected in ewes given 10^4 or 10^6 cfu. Anti-LLO and anti-IrpA antibodies were detected from day 15 in animals inoculated with 10^{10} cfu, and this strengthened the conclusion that these long-lasting shedders were infected but asymptomatic carriers. An anti-InlA response was detected only at a very low level. These results suggest that repeated daily doses are no more effective than a single dose in causing infection in ewes.
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Keywords: *Listeria monocytogenes*; Repeated dose; Ewe; Faecal excretion; ELISA

1. Introduction

The Gram-positive bacterium *Listeria monocytogenes* is widespread in nature and associated with severe disease (listeriosis) in both humans and animals (Low and Donachie, 1997). Infection is followed by production of circulating antibodies against virulence factors such as listeriolysin O (Berche et al., 1990), internalin A (Dramsi et al., 1997) and the internalin A-related protein (Grenningloh et al., 1997). These antibodies have been used for serodiagnosis of listeria infection in humans and in experimentally or naturally infected animals (Baetz and Wesley, 1995; Boerlin et al., 2003; Gholizadeh et al., 1996; Lhopital et al., 1993). Infection with *L. monocytogenes* is generally caused by consuming contaminated feed or food products (Slutsker and Schuchat, 1999), and it has been suggested that prolonged low daily doses

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increase the probability of listerial infection (Maijala et al., 2001; Roberts and Wiedmann, 2003). Sheep, cattle and goats frequently suffer from listeriosis, and some asymptomatic carriers shed *L. monocytogenes* in faeces and milk (Carrique-Mas et al., 2003).

Using oral inoculation of ewes as an experimental model, the aim of the present study was to compare the effects of *per os* inoculation with different doses in one or repeated administrations. The effects were evaluated from clinical records, bacteriological tests of milk and faeces, serological monitoring using different specific antigens, and contamination of the environment with the excreted bacteria.

2. Materials and methods

2.1. Listeria strain and inoculum

The *L. monocytogenes* F13 strain is a natural spectinomycin-resistant mutant derived from the LO28 strain; its virulence is equivalent to that of the LO28 strain in a mouse model (Lhopital et al., 1993). The inoculum cultivated on trypticase soy agar (TSA, BioMérieux, Marcy l'Etoile, France) was turbidimetrically adjusted to 4×10^{10} cfu ml^{-1} before appropriate dilution in phosphate buffered saline (PBS) and oral inoculation. For bacterial enumeration, the inoculum was serially diluted in PBS, and the number of cfu was counted on TSA plates.

2.2. Animals and experimental design

The use of animals complied with the European Directive 86/609/EEC (1986/11/24) and the European Directive 2000/54/EC (2000/09/04). Twenty-nine 18-month-old Préalpes ewes were randomly divided into 7 groups 15 days after their first lambing. They were reared in category 3 containment level facilities and fed *Listeria*-free pellets in order to prevent any spontaneous contamination with *Listeria*. Six groups of four animals were orally inoculated either once or daily for 10 days with 10^4, 10^6 or 10^{10} colony-forming units (cfu) of *L. monocytogenes* F13 (Lhopital et al., 1993). One group of five animals was kept as uninfected controls (Table 1). The animals and their environment were monitored for *L. monocytogenes* for 8 and 17 weeks, respectively.

Clinical signs and rectal temperature were recorded daily. Blood samples were taken before inoculation to check sera for the absence of anti-LLO antibodies using a dot-blot test (Lhopital et al., 1993), and then weekly until slaughter. Samples taken from the animals (blood, milk, faeces) and the environment (food, water from drinking bowls, slurry from pits) were checked for *L.*

Table 1
Effect of different doses and number of oral administrations of *L. monocytogenes* on faecal excretion and antibody responses to LLO and IrpA antigens of ewes, and on environmental contamination (drinking bowl water) in category 3 containment level facilities

	Ewe groups						
	0[a]	10^4[a]		10^6[a]		10^{10}[a]	
	0[b], 5[c]	1[b], 4[c]	10[b], 4[c]	1[b], 4[c]	10[b], 4[c]	1[b], 4[c]	10[b], 4[c,d]
Faecal excretion							
Maximal level (cfu g^{-1})	nd[e]	nd	nd	nd	<50	<50	≤150
Last excretion (day[f])	–	–	–	–	5[g]	13	17[h]
Drinking-bowl contamination							
Maximal level (cfu g^{-1})	nd	nd	nd	nd	≤5	nd	≤2500
Time (day)	–	–	–	–	62	–	90
LLO and IrpA ELISAs	nd	nd	nd	nd	nd	+[i]	+

Slurry from the common collecting pit was contaminated for at least 69 days.
[a] Oral daily doses (cfu).
[b] Inoculations (n).
[c] Ewes (n).
[d] One ewe died at day 9.
[e] Not detected.
[f] Day 0, day of inoculation for ewes given a single dose, and first day of inoculation for ewes given repeated daily doses for 10 days.
[g] Three of the four ewes shed *L. monocytogenes* once at day 1 after the first inoculation (and thus they did not differ from their counterparts with a single dose), the fourth shed once at day 5.
[h] Only one ewe shed *L. monocytogenes* beyond day 13.
[i] ODs above the cut-off values (LLO 0.37, IrpA 0.72).

monocytogenes twice a week for 2 weeks before inoculation, then every 2 days until day 20 post-inoculation, then twice a week, using a bacteriological enrichment test (AFNOR, 1997). Animals were slaughtered and necropsied between days 49 and 56, and samples (25 g each when possible) were aseptically collected from 14 organs.

2.3. Bacteriological examination

For isolation of *Listeria* we used a slightly modified enrichment method (AFNOR, 1997). A 25-g sample was incubated for 24 h at 30 °C in nine volumes of enrichment broth (half Fraser consisting of ($g\,l^{-1}$): proteose peptone 3 (Difco, Becton Dickinson, Meylan, France), 5; tryptone (Difco), 5; beef extract (Oxoid, Dardilly, France), 5; yeast extract (Difco), 5; sodium chloride (Merck, VWR International, Fontenay-sous-Bois, France), 20; $Na_2HPO_4{\cdot}2H_2O$ (Merck), 12; KH_2PO_4 (Merck), 1.35; lithium chloride (Sigma , Saint-Quentin-Fallavier, France), 3; esculin (Sigma), 1; ferric ammonium citrate (Merck), 0.5; acriflavin (Sigma), 0.0125; nalidixic acid (Sigma), 0.01. A 0.1-ml aliquot was then placed in 10 ml Fraser broth (AFNOR, 1997), which has twice the half Fraser concentrations of acriflavin and nalidixic acid, and was incubated for 24 and 48 h at 37 °C. From this subculture, 0.2 ml was plated on Oxford agar (AFNOR, 1997) consisting of ($g\,l^{-1}$): TSA, 40; esculin (Sigma), 1; ferric ammonium citrate (Merck), 0.5; lithium chloride (Sigma), 15; cycloheximide (Sigma), 0.4; colistin sulphate (Colimycine®, Aventis, Antony, France), 0.02; acriflavin (Sigma), 0.005; cefotetan (Apacef®, Astra Zeneca, Rueil Malmaison, France), 0.002; fosfomycin (Sigma), 0.01) and incubated for 24–48 h at 37 °C. *Listeria*-like colonies were substreaked on TSA and confirmed by examining the colonies under obliquely reflected light (McClain and Lee, 1988), and by the catalase reaction (positive), haemolysis of horse blood agar (BioMérieux), 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. After inoculation, blood, milk, faeces, water and slurry were cultured in the same way, with colistin in the Oxford agar replaced by 400 $mg\,l^{-1}$ spectinomycin (Sigma) (Lhopital et al., 1993).

To enumerate *Listeria*, 0.2 ml samples of pure blood, milk or water or of serially diluted food, faeces or slurry were plated on Oxford agar. After 24 and 48 h at 37 °C, *Listeria*-like colonies were counted, and some were substreaked on TSA and confirmed as described above.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Sera were screened with ELISAs using recombinant listeriolysin O (LLO), internalin A (InlA) and internalin A-related protein (IrpA) as *L. monocytogenes* specific antigens. The recombinant antigens LLO, InlA and IrpA (Darji et al., 1995; Lingnau et al., 1996) were kindly provided by Prof. J. Wehland (Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany). Purified LLO (L. Phan-Thanh, unpublished results adapted from Berche et al., 1990) was used to check the results obtained with recombinant LLO.

The ELISAs were performed in 96-well microtitration plates with flat-bottomed wells (Greiner, Solingen, Germany). Antigen was coated on plates with 100 μl of solution (1 $\mu g\,ml^{-1}$ in PBS) per well incubated overnight at 4 °C. The plates were washed three times with NaCl-Tween solution (0.05%, v/v Tween 20 in 9 $g\,l^{-1}$ NaCl). Serum samples (diluted to 1/100 in PBS) were then placed in the wells and incubated for 1 h at 37 °C. The plates were washed again, and peroxidase-conjugated anti-sheep immunoglobulin G (H + L) antibody (Jackson/Interchim, Montluçon, France) diluted 1/15,000 in PBS was added to the wells. After 1 h at 37 °C, the plates were washed and incubated with the chromogen 2,2′-azino-bis(3-ethylbenzothiazolin)-6-sulfonic acid (ABTS). After 1 h at room temperature, the absorbance was read at 414 nm (Multiskan RC, Labsystems, Cergy-Pontoise, France). The results were expressed as optical density (OD). The serum of infected sheep from Lhopital et al. (1993) was used as a positive reference, with titres of 3.09 for LLO, 3.16 for IrpA and 1.59 for InlA. Sera from animals with prolonged faecal excretion and high serological reactions after inoculation with 10^{10} cfu were taken 20 days after inoculation ($n = 7$) in order to define the cut-off values.

2.5. Definitions and statistics

Animals were considered active carriers if they excreted *L. monocytogenes* in their milk or in their faeces for more than 5 days following inoculation, 5 days being the maximum length of gastrointestinal transit (Ellis et al., 2002). Animals were considered infected if they were active carriers and positive to serological test. The serological results were analysed statistically using the Mann–Whitney or Kruskal–Wallis non-parametric tests (InStat 2 software, GraphPad Software, San Diego, CA, USA) and ROC analysis. ROC analysis was used for the selection of cut-off values and for the evaluation of the discriminatory power (area under the curve, AUC) of the

test (Greiner et al., 2000). ROC curves were calculated with Prism 4 software (GraphPad Software).

3. Results

3.1. Clinical examinations

The 24 inoculated ewes displayed no symptoms, except for a transient hyperthermia on day 2 in two animals that had been inoculated once with 10^{10} cfu. The mean of the rectal temperatures was higher in the ewes inoculated once with 10^{10} ($P < 0.05$). One animal died on day 9 after presenting with non-listerial mastitis on day 5 and listerial bacteraemia on day 7. *L. monocytogenes* of the inoculated phenotype were isolated from all organ ($n = 14$) samples collected at necropsy (Table 1).

3.2. Bacteriological examinations

No *Listeria* was found before inoculation in either animals or in the containment facilities. No *Listeria* was recovered after inoculation in blood (except the one listerial bacteraemia mentioned above), milk and food.

L. monocytogenes was found from day 1 post-inoculation in the faecal samples of the eight animals inoculated with 10^{10} cfu, this number decreasing from day 9 to day 17 (Table 1 and Fig. 1A). *L. monocytogenes* was found on day 1 in the faeces of three of the animals given repeated 10^6 cfu doses and on day 5 in one of them, which is equivalent to the length of gastrointestinal transit. Therefore, only those eight animals excreting *L. monocytogenes* for more than 5 days post-inoculation were considered active carriers. *L. monocytogenes* excretion level remained low (<150 cfu g^{-1}), whereas the environment (drinking bowls, pits) was more contaminated (<2500 cfu g^{-1}). Animals were slaughtered and necropsied between days 49 and 56 with no *Listeria* recovered, whereas the environment in the containment facilities remained contaminated until day 69 (slurry) and 90 (bowls) post-inoculation (Table 1).

3.3. Serological examinations

The average level of anti-InlA antibodies in the ELISA was higher ($P = 0.0001$) in infected animals (0.24 ± 0.04 OD_{414}) than in healthy animals (0.18 ± 0.04 OD_{414}) (Fig. 2). However, that difference was estimated too small to be interpreted as a useful criterion of infection.

The results obtained with purified LLO and recombinant LLO in the ELISA for screening the sera of

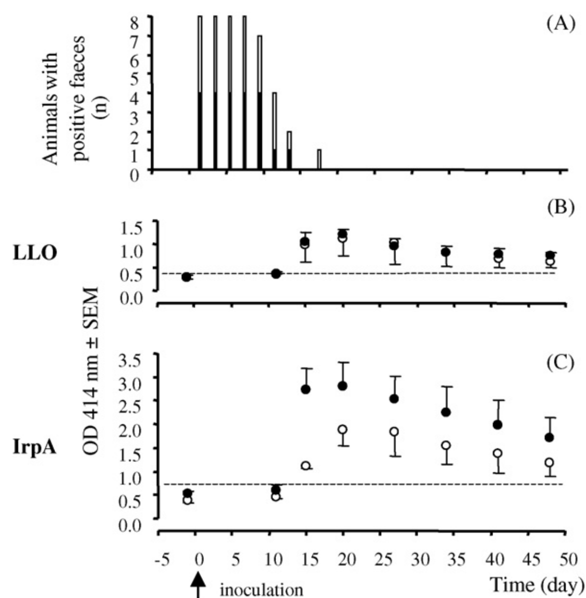

Fig. 1. Evolution of the number of ewes shedding *L. monocytogenes* in faeces (A) and of their circulating anti-LLO (B) and anti-IrpA (C) antibodies, measured by ELISA. These eight ewes were inoculated *per os* with 10^{10} cfu *L. monocytogenes* once at day 0 (black bars and circles) or daily from day 0 to day 10 (open bars and circles). InlA response was too low to be interpreted (see text). Day 0: first inoculation day. OD 414 nm ± S.E.M., the ELISA optical densities at 414 nm are means ± S.E. of the means. Dotted lines: cut-off values.

experimentally inoculated ewes were not distinguishable. The anti-LLO response was detectable in the active carriers only. Ewes given 10^4 and 10^6 cfu (either once or repeatedly) developed no detectable seroconversion, unlike those given 10^{10} cfu, which showed moderate seroconversion from day 11 post-inoculation. There was no difference in the LLO response of ewes given a single 10^{10} cfu dose and those given repeated 10^{10} cfu doses (Fig. 1). The ELISA cut-off values for LLO (0.37) and IrpA (0.72) were defined by an ROC curve analysis of the seven active carriers (without counting the ewe which

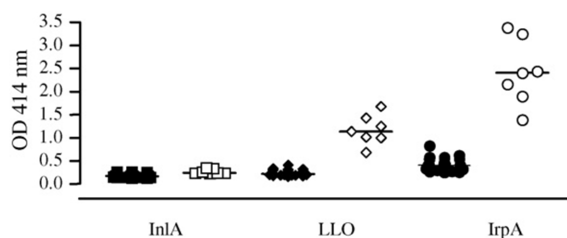

Fig. 2. Individual anti-InlA, -LLO and -IrpA antibodies titres, measured by ELISA in sera of the ewes: 29 *Listeria*-free controls (black symbols), seven ewes inoculated with 10^{10} *L. monocytogenes* (open symbols) on day 20 post-inoculation. One infected ewe died at day 9, see text. OD 414 nm, ELISA optical densities at 414 nm. Bars, OD median values.

died before day 20) and the 29 *Listeria*-free animals before inoculation (controls).

The IrpA ELISA showed no seroconversion in the non-excreting ewes inoculated with low doses of bacteria (10^4 or 10^6 cfu). However, the active carriers, i.e. those given 10^{10} cfu, showed a surge in anti-IrpA titre from day 15 post-inoculation, which was twofold that of anti-LLO (Fig. 1). There was a smaller increase in the anti-IrpA titre and a lower maximum in ewes given repeated 10^{10} cfu doses than in their counterparts given a single dose ($P = 0.0059$). There was generally a delay of at least 14 days between the start of faecal shedding of *L. monocytogenes* (from day 1 post-inoculation) and a significant increase in serum anti-LLO or anti-IrpA antibodies (from day 15 to day 21). The anti-IrpA titre was still high on day 48, 1 day before slaughter (Fig. 1).

4. Discussion

These results confirm a dose-effect. Using small groups of ewes in our conditions, even high oral doses did not produce regular and acute clinical effects. Repeated daily doses increased the duration of detectable faecal excretion but were no more effective than a single dose in causing a serological reaction. Serological responses were detected mainly against purified and synthetic LLO antigens and IrpA antigens.

While sheep as a species are naturally susceptible to *Listeria* infection, the relevance of the experimental model, especially the high doses, can be questioned. When silage is contaminated with *L. monocytogenes*, bacterium distribution is uneven (Fenlon, 1999). Therefore, silage-fed ewes can ingest either a single dose, or repeated daily doses over a period of time, especially when using a mixing and unloading trailer. It has been suggested that even low levels of *L. monocytogenes* in food should be avoided because prolonged low daily doses increase the probability of listerial infection in humans (Maijala et al., 2001). In the present study, the 10-day period of daily administration was chosen because it is comparable to the median period (8 days) between sheep being fed spontaneously contaminated silage and the appearance of the first clinical signs (Grønstøl, 1979; Low and Renton, 1985).

Healthy animals generally keep well after oral inoculation (Lhopital et al., 1993; Low and Donachie, 1991), and this was the case in our experiment where transient hyperthermia was only significant in the once-inoculated 10^{10} cfu group. However, one animal died during the experiment (Table 1). Some factors, such as concurrent disease, may predispose animals to infection with pathogenic *Listeria* (Roberts and Wiedmann, 2003). This was probably the case here, since only the animal that died presented with non-listerial mastitis, leading to a serious systemic infection with *L. monocytogenes*.

The 10^4 cfu dose, even when repeated daily, as well as the 10^6 cfu dose, appeared to have no effect on faecal excretion. The repeated 10^6 cfu dose did not increase or prolong *L. monocytogenes* faecal excretion beyond the mean residence time of undigested feed residues in the gastrointestinal tract, i.e. the normal gastrointestinal transit (Ellis et al., 2002). Consequently, *Listeria* recovered beyond that time were probably attached to undigested residues. These results taken overall suggest that the ewes given 10^4 or 10^6 cfu (repeated or not) remained passive carriers. On the other hand, the ewes given 10^{10} cfu were considered as asymptomatic active carriers, also called healthy carriers (Casadevall and Pirofski, 2000), excreting in their environment for 2 weeks. This is the first demonstration that ewes could in fact be long-lasting healthy carriers and faecal shedders of *L. monocytogenes*, because no spontaneous contamination from outside was detected in our category 3 containment level facilities, and all the *Listeria* isolates were indistinguishable from the phenotype of the inoculated *Listeria* strain.

In addition to active carriage, *Listeria* infection was estimated by serology using *L. monocytogenes*-specific antigens (Berche et al., 1990). The major virulence factor LLO is a well-documented reference *L. monocytogenes* antigen (Wesley, 1999) used with human (Berche et al., 1990), bovine (Baetz and Wesley, 1995), caprine (Miettinen and Husu, 1991) and ovine (Low et al., 1992) sera. InlA is an internalin involved in the virulence of *L. monocytogenes* (Vazquez-Boland et al., 2001) and unlike any known protein (Dramsi et al., 1997; Gaillard et al., 1991). The internalin A-related protein IrpA is associated with the virulence of *L. monocytogenes*, and is also called internalin C (Engelbrecht et al., 1996; Lingnau et al., 1996). Like LLO, IrpA is a major protein target of the human humoral response to *L. monocytogenes* (Grenningloh et al., 1997). Thus LLO, InlA and IrpA can all be considered to be specific antigens for detecting *L. monocytogenes* infection in ewes.

Our results suggest that repeated low daily doses did not facilitate seroconversion, even when animals excreted *L. monocytogenes* (10^6 cfu). This observation confirms the indication from the bacteriological results that low daily doses did not induce infection. Moreover, the anti-IrpA titre level in ewes given repeated 10^{10} cfu doses was lower than that of ewes given a single 10^{10} cfu dose. To date, this finding has not been reported in experimental listeriosis. This result could suggest that a partial

tolerance was induced by repeated doses of antigen, probably facilitated by the oral route of access to the immunological system (Weiner, 2001).

Anti-InlA antibodies were detected at very low titres at 10^{10} cfu doses only. This finding could be explained by the fact that the challenge F13 strain is derived from the LO28 strain (Lhopital et al., 1993) shown to produce a truncated InlA that is secreted and not attached to the bacterial cell envelope (Jonquières et al., 1998). However, our result is consistent with a previous report showing that anti-InlA antibodies were not detected in the sera of five listeriosis patients, while anti-LLO antibodies were detected in all cases (Dramsi et al., 1997).

As the host immune status represents the most important host component in the pathogenesis of listeriosis (Farber and Peterkin, 1991), any factor that weakens the immune system will probably increase the likelihood of infection (Maijala et al., 2001; Roberts and Wiedmann, 2003). Most human listeriosis cases occur in immunocompromised individuals (Farber and Peterkin, 1991), but no such data are available for animals. Stress factors such as bad weather, transport, or concurrent disease may predispose animals to listerial infection (Wesley, 1999). However, studying such predisposing factors was not an aim of our work. On the other hand, as our ewes were raised in a *Listeria*-free environment and their sera checked for antibodies to LLO before inoculation, they had acquired no resistance to *L. monocytogenes* (Miettinen and Husu, 1991). Therefore, from our observation of a single case of listeriosis following non-listerial mastitis, but no effect of repeated low daily doses compared to single doses, we can conclude that daily doses did not increase listerial infection in ewes under our conditions and according to the criteria monitored

The *L. monocytogenes* infection eventually cleared, since the bacteria were not found at slaughter. Again, because no spontaneous contamination from outside was detected in our category 3 containment level facilities, the survival of *L. monocytogenes* in the environment (Fenlon, 1999; Roberts and Wiedmann, 2003) was effectively confirmed: *Listeria* contamination was detectable, i.e. persisted for 2–3 months, even after the ewes had been slaughtered. Overall, our results suggest that the level of the dose was more important than its repetition in infecting ewes.

In conclusion, *per os* repeated daily doses of *L. monocytogenes* compared to single doses did not increase listerial infection in ewes under our conditions in terms of faecal shedding or anti-LLO, -IrpA or -InlA humoral responses.

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