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A combination of diagnostic tools for rapid screening of ovine listeriosis

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Abstract

A combined serological and PCR method for the detection of *Listeria monocytogenes* infection in symptomatic and asymptomatic ovine flocks was evaluated. Seventy-eight milk samples and 157 serum samples were analysed using a *L. monocytogenes* PCR detection kit and an anti-listeriolysin O IgG immunoassay kit. The combined use of these commercial kits allowed a rapid and effective detection of *L. monocytogenes* infection in both the early stage, before seroconversion, and in a later phase, even after antibiotic therapy.

Keywords: PCR; ELISA; Ovine listeriosis; *Listeria monocytogenes*; Listeriolysin O; Epidemiological survey

1. Introduction

Listeria monocytogenes is the causative agent of listeriosis, a severe disease associated with a high case fatality rate. Nearly all the domestic animals are susceptible to *Listeria* infections, but animal listeriosis most commonly occurs in ruminants (Cooper and Walker, 1998). In general, the disease is more frequent in winter and early spring and it has been associated with silage feeding (Farber et al., 1988; Husu, 1990).

The main clinical features of ruminant listeriosis are encephalitis, septicemia, abortion and mastitis (Low and Linklater, 1985; Low and Donachie, 1997), although healthy carriage of *L. monocytogenes* has also been amply described in a variety of animal species (Low and Donachie, 1997). In particular, udder infection may be subclinical, with apparently healthy animals excreting the pathogen with the milk for long periods of time (Fthenakis et al., 1998; Wagner et al., 2000).

L. monocytogenes is a zoonotic agent, and decisive role in the prevention of food-borne listeriosis in humans is the reduction of the presence of *L. monocytogenes* in all the critical stages of the food production and the distribution chain, including the epidemiological surveillance of livestock (Anonymous, 2003a,b). Thus, sensitive and specific tests to identify *L. monocytogenes*-infected animals are of great importance in carrying out epidemiological surveys to develop appropriate control strategies.

Bacteriological testing (Quinn et al., 1999; Walker, 1999) and histological examination are the classical methods used for the laboratory diagnosis of listeriosis in animal specimens. These procedures are usually considered the "gold standard" to which other methods are compared. However, these protocols are time consuming and require several manual steps to be completed (Gronstol et al., 1986). Isolation of *L. monocytogenes* may be unsuccessful even when adequate samples are examined and culture-negative cases can be the result of few or no bacteria in the lesions

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examined (Barlow and Mcgorum, 1985; Johnson et al., 1995) and in case of antibiotic treated animals. In addition, the histopathological analysis requires post-mortem clinical samples, therefore it depends on the availability of samples and the fatality rate in the course of the disease.

According to these premises, rapid and sensitive methods for the diagnosis of animal listeriosis and the detection of subclinical infections are needed. This study was undertaken to evaluate the combined use of two commercial kits: an enzyme-linked immunosorbent assay (ELISA) (Giammarini et al., 2004) for the recognition of specific antibodies, and a PCR-based method (Amagliani et al., 2004) for the direct detection of *L. monocytogenes* DNA in milk samples.

Field serum and milk samples from symptomatic and asymptomatic ovine flocks were analysed with the two kits, and the resulting data were compared with those obtained from bacteriological testing and histological examination. The possibility of using the two kits as mass-screening diagnostic tools in epidemiological studies is discussed.

2. Materials and methods

2.1. Samples collection

From October 2003 to May 2004, milk and serum samples were collected from five different flocks, after clinical examination. The flocks were located in Central Italy, some in the Appennini area, and one (flock B) closer to the Tirrenian sea. In the flock identified as A, in which several animals had shown neurological signs suspect of listeriosis (''circling disease'') and six animals died, two sets of samples were collected. Serum and milk samples were first collected in October 2003, 2–3 weeks after the occurrence of the disease; the sampling was repeated three months later (January 2004). After the diagnosis of listeriosis, the animals were treated with ampicillin (10 mg/Kg), by administrating IM a long-acting product twice in 48 h. Sheep belonging to flock B (eight dead), showed similar neurological symptoms and were sampled for cultural (milk) and serological tests, few weeks after the occurrence of the disease. Animals from flocks identified as C, D and E had never shown any neurological or other clinical signs indicative of listeriosis. Samples of milk and serum were collected also in flocks C and D. Flock E was tested twice in seven months; however, due to the absence of lactating ewes, only serum samples were collected for serological testing. Neverthless, four animals from this flock were sacrificed, and mammary tissue and lymph nodes were collected for microbiological examination. A summary of samples collection is shown in Table 1.

Blood samples were taken at random by jugular venipuncture and sera were obtained by centrifugation at 3000*g* for 15 min. Milk samples for PCR analysis and culture were collected aseptically from the same animals. Each sample was marked with the sheep's identification number and sent to the laboratory in refrigerated conditions.

Table 1
Sample collection scheme, with number of examined and positive samples for each assay

Flock	No. of sheep in flock	Clinical suspect	Sampling date	Milk samples		Serum samples
				Culture[a]	PCR[a]	ELISA [a]
A	350	+	October 2003	n.d.	7/15	2/24
A		+	January 2004	0/23	2/23	20/23
B	1000	+	March 2004	0/20	n.d.	17/20
C	500	–	March 2004	0/20	0/20	2/20
D	600	–	May 2004	0/20	0/20	2/20
E	100	–	October 2003	n.d.	n.d.	0/30
E		–	May 2004	n.d.	n.d.	2/20
Total	2550			83	78	157

n.d., not determined.
[a] Number of positive results/total number of examined samples.

2.2. Microbiological analysis

The microbiological procedure used for the detection of *L. monocytogenes* in milk from flocks A, B, C, D and in clinical samples from flock E was performed following the standard protocol adopted and validated at the ''Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche'', according to ISO 10560/1993 (Anonymous, 1993).

Briefly, samples were mixed keeping a ratio 1:10 v/v in Listeria Enrichment Broth Base with Listeria Selective Enrichment Supplement (LEB) for milk and in Fraser broth for mammary tissues, and incubated at 30 °C for 48 h. Subsequently, 0.1 ml were streaked onto Listeria Selective Agar Base (Oxford) plates with Listeria Selective Supplement (Oxford), and incubated at 37 °C for 48 h. Presumptive *Listeria* colonies were subjected to biochemical (API Listeria, bioMérieux, France) and serological (Listeria Rapid Test, Oxoid, UK) confirmation tests. All culture media were purchased from Oxoid.

2.3. Histopathological examination

The diagnosis of listeriosis was based on the microscopic assessment of fixed tissues of two dead sheep from clinically suspected flocks. The necropsies were performed within 24 h after death. For the histological examination, CNS tissue samples were selected and fixed in neutral 10% formalin and transverse sections were taken at the level of the pons cerebri and medulla oblongata. Samples were dehydrated, embedded in paraffin wax and 4–5 μm-thick sections were stained with Haematoxilin and Eosin (H–E).

2.4. DNA extraction

Ten milliliters-milk samples were centrifuged at 6000*g* for 20 min at 4 °C and the pellets were subjected to DNA extraction with paramagnetic nanoparticles using the *L. monocytogenes* DNA Isolation Kit: Milk (Diatheva, Italy). As indicated by the manufacturer, bacterial cells, if present in the pellet, were lysed, treated with lysozyme, RNase A and proteinase K, then the mixtures were gently shaken with

the magnetic particles in presence of the specific DNA-binding buffer. The bound DNA was washed, eluted from the beads by the addition of sterile water and then alcohol-precipitated. Finally, each sample was dissolved in sterile water.

2.5. DNA amplification and PCR product detection

The DNA samples obtained from milk by magnetic extraction were amplified with the *L. monocytogenes* PCR Detection Kit (Diatheva), according to manual instructions. For each reaction, 10 µl of the extracted samples were added. The PCR mixture contained an internal control revealing any inhibition within the reaction. In addition, the functionality of PCR was monitored by the presence of both a positive and a negative control for each amplification round.

A 25 µl volume of the PCR products was visualized by electrophoresis in a 2% (w/v) agarose gel, stained with ethidium bromide. A low molecular weight DNA standard (ΦX174 DNA *Hae*III, Roche, Germany) was analysed together with the samples. Amplicons were visualized and analysed with a Gel Doc 2000 apparatus using Quantity One Quantitation Software (Bio-Rad, California, USA).

2.6. Serology

Serum samples were tested for the presence of specific *L. monocytogenes* antibodies using the sheep anti-LLO IgG Immunoassay kit (Diatheva) according to the manufacturer's instructions. The diluted sera (1:100) were tested in duplicate on microtitre strips coated with the listeriolysin O (LLO) antigen. The antigen–antibody complex was detected by adding anti-IgG HRP-conjugated globulin, and revealed by incubating the strips with the chromogen solution. Absorbance was measured at 405 nm by an ELISA microwell plate reader. Each sample was classified as positive, negative or equivocal by interpreting its mean absorbance value as indicated in the datasheet supplied with the kit.

2.7. Statistical analysis

Differences among the flocks in the qualitative response obtained by ELISA assay were evaluated using the chi square test (χ^2) in contingency tables. The divergencies between the seroprevalent and the non-seroprevalent flocks in the observed frequencies of positive and negative samples were compared and p values <0.001 were considered to evaluate the statistical significance. Equivocal results were included as negative in the analysis (Bas et al., 2001).

3. Results

3.1. Clinical, microbiological and histological examinations

Five flocks were considered in this study: flocks A and B, with suspect clinical signs of listeriosis (neurological symptoms) and flocks C, D, E, without any clinical evidence of the disease (Table 1).

Milk samples from flocks A January 2004, B, C, and D and clinical samples from flock E were examined by culture with negative results.

In flock A October 2003, culture was not carried out because of an insufficient amount of milk. However, tissue samples from dead sheep of this flock were subjected to histological examination. The brain tissues showed perivascular mononuclear cuffing, focal gliosis and single or multiple microabscesses characterized by neutrophil infiltrations.

3.2. PCR analysis

The magnetic DNA extraction followed by PCR amplification used in this study is a direct detection method that amplifies *L. monocytogenes* DNA from milk samples with no need of an enrichment step. It yields a 172 bp band indicating the presence of the bacterial target sequence (Amagliani et al., 2004), and a 112 bp product, corresponding to the internal control of the amplification reaction. This method yielded a 47% positivity rate for flock A, October samples, and a 8.7% for samples collected in January in the same flock. No amplification products of expected size were obtained from the samples collected in the flocks (C and D), where clinical signs of listeriosis were not observed (Table 1). In all cases, gel analysis showed the presence of the internal control, indicating the absence of PCR inhibitors in the extracted samples used as PCR templates (Fig. 1).

3.3. ELISA

Detection of anti-LLO antibodies by the commercial ELISA was carried out for a total of 157 serum samples obtained from both symptomatic and asymptomatic sheep. Eight percent of the samples from flock A resulted positive at the first analysis (October 2003), while the percentage increased to 87% after 3 months (January 2004). A high number of positive responses (85%) was also found in flock B. On the other hand, flocks C, D and E showed positivity rates ranging from 0% to 10% (Table 1).

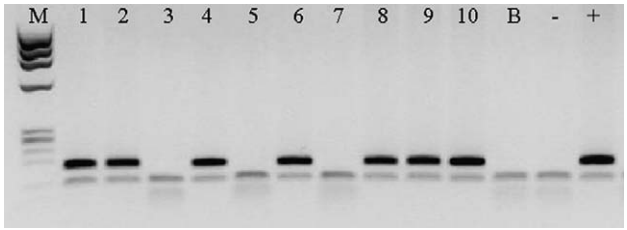

Fig. 1. PCR analysis of 10 milk samples from flock A (October 2003 sampling). DNA was extracted using the *Listeria monocytogenes* DNA Isolation Kit: Milk and amplified by the *Listeria monocytogenes* PCR Detection Kit. M, molecular weight marker ΦX174 DNA/*Hae*III, (Roche); lanes 1–10, milk samples; B, extraction negative control; −, PCR negative control (no DNA); +, PCR positive control.

The frequency of positive results obtained by the immunoenzymatic assay allowed the separation of the flocks into two distinct groups: the seroprevalent group (SP), corresponding to flock A, January 2004, and flock B; the non-seroprevalent group (NSP) corresponding to C, D and E (October 2003 and May 2004 samplings), in which the rate of seropositive animals was always quite low and never exceeded 10%. Flock A, October 2003, which presented clinical symptoms but showed a low seropositivity rate, was considered as exposed but non-seroprevalent (ENSP) and was compared with the two groups above. Statistical analysis revealed significant differences ($p < 0.001$, confidence level of 99.9%) between flocks SP and NSP ($\chi^2 = 178.66$) and between ENSP and SP flocks ($\chi^2 = 37.84$), while no significant difference ($\chi^2 = 0.0805$) was observed between ENSP and NSP flocks.

4. Discussion

International regulative Agencies aim at the prevention of food-borne infections through the reduction of the incidence of zoonosis. The proposed control plans involve the assessment of the animals' health status and the implementation of strategies which continuously monitor the presence of food-borne pathogens in animals, including *L. monocytogenes*. In this perspective, the epidemiologic screening of ovine flocks could be a helpful preventive measure, especially if rapid and sensitive diagnostic procedures are employed. Diagnosis of animal listeric infection, currently achieved by microbiological or histological tests, presents the disadvantage of being laborious and time consuming. Therefore, molecular techniques are increasingly being used as new alternative faster diagnostic methods, with enhanced sensitivity and reproducibility.

In this study, we evaluated the usefulness of a combination of PCR and ELISA tests as a diagnostic mass-screening tool. The presence of *L. monocytogenes* infection was investigated in symptomatic and asymptomatic sheep flocks together with the use of conventional techniques. The molecular assays were carried out with two commercial kits detecting a species-specific DNA sequence from the *hlyA* gene, and anti-LLO IgG, respectively. Indeed, LLO is a virulence determinant of *L. monocytogenes* (Cossart and Portnoy, 2000) and the encoding *hlyA* gene has been employed by several Authors as a molecular target for its detection in PCR assays (Choi and Hong, 2003; Amagliani et al., 2004). Our PCR analysis revealed the presence of *L. monocytogenes* in milk samples from infected sheep through the amplification of the above-mentioned 172 bp specific DNA fragment. The specificity of the assay was validated in a previous study (Amagliani et al., 2004) and confirmed by testing with negative results a strain of *L. ivanovii* subsp. *ivanovii* (DSM 20750) possessing a similar *hly* gene (data not shown). The detection limit of the assay was experimentally determined and resulted in a very high sensitivity (2 cfu), suitable for this kind of application (*L. monocytogenes* PCR Detection Kit datasheet). This method, in association with the magnetic DNA extraction from milk, provided a rapid diagnosis with no need of an enrichment step. The presence of an internal amplification control ruled out the possibility of false negative results due to the presence of PCR inhibitors in the samples. This molecular assay detected the infection successfully even when the cultural method failed. The negative results obtained in the symptomatic flocks (A and B) with the microbiological assay could be justified by either a very small contamination rate or the absence of living bacteria, due to the administration of antibiotics. However, the lesions examined by histopathology were considered compatible with the clinical suspect of listeriosis (Jubb and Huxtable, 1993). Furthermore, the antibiotic therapy, administered to the sheep of flock A between the two samplings, could have determined a decrease of positive PCR results in the second test carried out on those animals.

LLO is also a target of the anti-*Listeria* immune response (Berche et al., 1990) and numerous studies have demonstrated the potential of this protein as an antigen for serological assays. Antibodies to LLO have already been identified in the serum of goats (Bourry et al., 1997), sheep (Lhopital et al., 1993), lambs (Low and Donachie, 1991), cows (Bourry and Poutrel, 1996) and humans (Berche et al., 1990) by Western blot, dot-blot or ELISA analyses.

The serological assay evaluated in this study identified infected sheep in both the flock A (January 2004) and B, in a percentage ranging from 87% to 85% of the animals. A different serological scenario was observed in non-symptomatic flocks, where the rate of positive animals did not exceed 10%. These positive reactions could be due to occasional contacts of the animals with the pathogen, due to its wide distribution in the environment (Nightingale et al., 2004). According to these results and to other previous experimental observations with field samples (unpublished results), a threshold value of 10% positivity was arbitrarily assigned, and flocks with a positivity rate higher than this value have been considered as seroprevalent, and therefore as infected. However, in the case of a positivity rate lower than or equal to 10% and a positive PCR assay, a subsequent ELISA test was necessary to identify the infectious status after the seroconversion (see flock A Oct 2003).

Statistical evaluation revealed that the infected flocks examined during the "window period" gave results not significantly different from the non-infected flocks. This indicates that an active infection cannot be diagnosed in its very early stage, because of the low level of circulating antibodies.

With respect to the conventional procedures, the two molecular methods presented a significantly shorter analysis time, and possibly a higher sensitivity. Moreover, their combined use may counterbalance their limitations. In flock A, in the early phase of the infection we observed frequent PCR positive reactions due to the shedding of bacteria in the milk but the corresponding serum samples were generally ELISA negative. On the contrary, several

weeks later and after antibiotic treatment of the animals, a lower PCR positivity rate and a high seroprevalence rate were observed. Such findings also underline the fact that the serological test alone is not sufficient for the diagnosis of ovine listeriosis. However, it can still be considered a useful tool for a quick and not expensive processing of large number of samples.

In conclusion, although the isolation of *L. monocytogenes* remains an important criterion for the diagnosis of listeriosis, the combined use of ELISA and PCR can enhance the overall sensitivity of the diagnostic procedure and could represent an effective approach to the epidemiological screening of ovine flocks.

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