

Epidemiological and diagnostic aspects on listeriosis in sheep

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Abstract

Listeria monocytogenes is one of the most important bacterial pathogens for sheep and goat. It infects central nervous system as well as genital system causing meningoencephalitis, nervous signs and abortion. Thirty three sheep blood samples (3 of which showed clinical signs of listeriosis as twisting of neck and moving in circles with torticollis and incoordination) were examined by the real-time PCR for lesteriosis. The brains of the affected cases were examined bacteriologically after death of these animals. The contact animals (30 asymptomatic cases) showed negative results by the real-time PCR. The PCR methods showed positive results with the three samples which collected from 3 symptomatic sheep while culture method gave positive results only with two samples.

Key words: *Listeria monocytogenes*, meningoencephalitis, culture, PCR.

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 and Donachie, 1997). Sheep, cattle and goats often shed *L. monocytogenes* in their faeces without symptoms (Wesley, 1999).

Animals themselves and the environment considered the sources of contamination for human foods such as milk (McLauchlin, 1997). Since *L. monocytogenes* mastitis is rare, raw milk is mainly contaminated from the environment, probably by faeces when farming and milking procedures are carried out under conditions of inadequate hygiene (Sanaa *et al.*, 1993).

Listeria infection causes clinical syndromes of abortion or neonatal septicemia, encephalitis localized to the brain stem or spinal cord is the most common illness of adult animals (Blood

et al., 1989). Ovine encephalitic listeriosis is generally characterized by rapidly progressive clinical disease, and flocks commonly suffer mortality higher than that observed in cattle. (Bakulov and Kotliarov, 1966; Gitter *et al.* 1986; Lippman, 1969). Most ovine outbreaks are associated with ingestion of silage (Cleff and Blandford, 1965; Gitter *et al.*, 1986).

To enhance the diagnosis accuracy, a serodiagnosis assay based on the detection of serum antibodies directed against listeriolysin O (LLO) has been proposed (Berche *et al.*, 1990). However, it allows only a retrospective diagnosis for listeriosis and lacks specificity, since a false-positive result can be due to cross reactions with hemolysins from other Gram-positive bacteria. Nucleic acid amplification testing by real-time PCR assay is a rapid diagnostic procedure, and it has been used successfully to diagnose a wide range of central nervous system infections (Corless *et al.*, 2001). Several molecular methods have been devel-

oped in the food industry (D'Agostino *et al.*, 2004; Gouws and Lidedemann, 2005), based on the amplification of several specific genes of *L. monocytogenes* (*iap*, *hly*, *prfA*, *actA*) (Hein *et al.*, 2001). Some of these assays are sensitive enough to be valuable for the detection and quantification of *L. monocytogenes* in the environment and food products (Gouws and Lidedemann, 2005; Hein *et al.*, 2001). However, the results are highly dependent on the method used, the chosen target to be amplified, and the complexity of the food matrix product. Although rapid, specific, and sensitive detection of *L. monocytogenes* is important for medical diagnosis, only a few works have described the application of these tests for the diagnosis of CNS listeriosis in animals (Greisen *et al.* 1994 and Jatou *et al.*, 1992).

The aim of the present study is to throw the light on PCR as rapid, recent and confirmatory technique for diagnosis of listeriosis in sheep.

Material and Methods

1. Samples Collection:

From Kalyobia governorate three blood samples were collected from sheep showing nervous clinical signs (circling disease). Also, the brain tissue were collected from them after death to isolate the microorganism. 30 blood samples were collected from asymptomatic contact animals. Each sample was marked with sheep's identification number and sent to the laboratory in refrigerated conditions.

2. Isolation of *listeria monocytogenes* (OIE, 2008)

Bacteriological isolation was attempted from hind brain (medulla oblongata) of the three clinical affected sheep. 10-25 grams of affected brain sample was inoculated into listeria enrichment broth then incubated at 30 °C for 48 hours. A loopful from the previously incubated media was streaked onto Oxford agar plates and incubated at 37 °C for 24-48 hours. 3-5 Colonies showing typical morphological characters of listeria (dew drop-like, black colonies with brown hallow or dark brown, 1-2 mm in diameter) were picked up and reinoculated on-

to trypticase soya agar supplemented with 0.6% yeast extract (TSA-YE) and incubated at 30 °C for 24 hours. The obtained colonies were incubated into semisolid agar medium and kept in refrigerator at 4 °C for further identification.

3. Real time PCR for the *hly* gene

Extraction of DNA from blood samples

L. monocytogenes-DNA was extracted in parallel to bacteriological examination from blood and tissue sample from affected brain and from blood samples of in contact animals using High Pure PCR Template Preparation Kit (Roche, Diagnostic GmbH, Mannheim, Germany) according to manufacturer's instruction. Purified and concentrated template DNA was eluted in elution buffer and preserved at -20 °C till subjected to PCR procedure.

Primer and probe design for *hly* gene:

The target DNA consisted of a well-conserved region of the single gene *hly* encoding listeriolysin O, a thiol-activated pore-forming cytolysin (Kayal and Charbit, 2006). The primers were designed according to (Le Monnier *et al.*, 2011). The design of the primer was conducted after alignment of the sequences of various cytolysins published in GenBank with the *hly* gene of *L. monocytogenes*: the pneumolysin (PLY) of *Streptococcus pneumoniae*, the perfringolysin of *Clostridium perfringens* (PFO), the streptolysin of *Streptococcus pyogenes* (LSO), the ivanolysin O of *Listeria ivanovii* (IVO), and the seeligeriolysin O of *Listeria seeligeri* (LSO) (NCBI sequence navigator). The forward primer LmH.172F (5'-TT TCA TCC ATG GCA CCA CC-3') and the reverse primer LmH.242R (5'-ATC CGC GTG TTT CTT TTC GA-3') were used to amplify a 71-bp fragment. The amplicon was detected with a TaqMan internal oligonucleotide probe, LmH.199T (FAM-5'-CGC CTG CAA GTC CTA AGACGC CA-3'-TAMRA). The primers were synthesized by BioTez-Berlin-Buch GmbH, Berlin, Germany. The probe synthesized by Eurogentec S.A., Seraing, Belgium.

Protocol of real-time PCR

Real-time PCR targeting *hly* gene used as rapid, direct method for detection of *L. monocyto-*

gene in clinical samples. PCR was carried out in 25 µl reaction volume. The mastermix was prepared on ice block in UV clean Lab. PCR mixtures contained 1 µl of LmH-172 forward primer (10 pmol/µl), 1 µl of LmH-242 reverse primer (10 pmol/ µl), 0.75 µl TaqMan probe-LmH-199T (FAM-10 pmol/µl), 2.25 µl RNase free water and 10 µl QuantiTect PCR MasterMix (Qiagen). Finally, 5.0 µl template was added. The PCR reaction was performed in Stratagene thermocycler (In central Lab. Of Faculty of Veterinary Medicine, Suez canal University)with the following program: Initial denaturation and activation of Taq-polymerase for 15 min at 95°C followed by 45 cycles of 15 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C then cooling for 30 sec at 40°C.

Results

1 Clinical findings

Clinical course of disease was characterized by circling movements. In initial stages, animals were moving aimlessly away from the herd with slightly deviated neck. With progression of disease, twist in the neck increased and animal started moving in circles with torticollis and incoordination see photo 1. In the advanced stages of illness, animals became recumbent, showing convulsions and paddling movements. Ultimately animal were lying with their neck turned towards flank. At this stage, animals were completely anorectic and unable to rise. The course of disease varied from 4 to 7 days after appearance of first clinical symptom(s) see photo 2.



Figure (1). Sheep infected with *Listeria monocytogenes* showed torticollis as in photo 1, then became recumbent and turned head as in photo 2.

2. Bacteriological findings

Two isolates were obtained from the hind parts of brain of recently dead animals. Typical colonies showed dew drop-like, black colonies with brown hallow or dark brown, 1-2 mm in diameter. The culture showed one negative animal despite of appearance of clinical signs.

3. PCR analysis

Real-time PCR analysis did not provide false-negative result in any of the analyzed cases

when *L. monocytogenes* was isolated by standard culture of brain samples. The real-time PCR showed positive result with the three samples of clinically affected animal either with blood or brain tissue samples, see figure 2. There is no detectable ct-value in PCR result for in contact animal without CNS involvement (bacteremia or nervous signs), see figure 2.

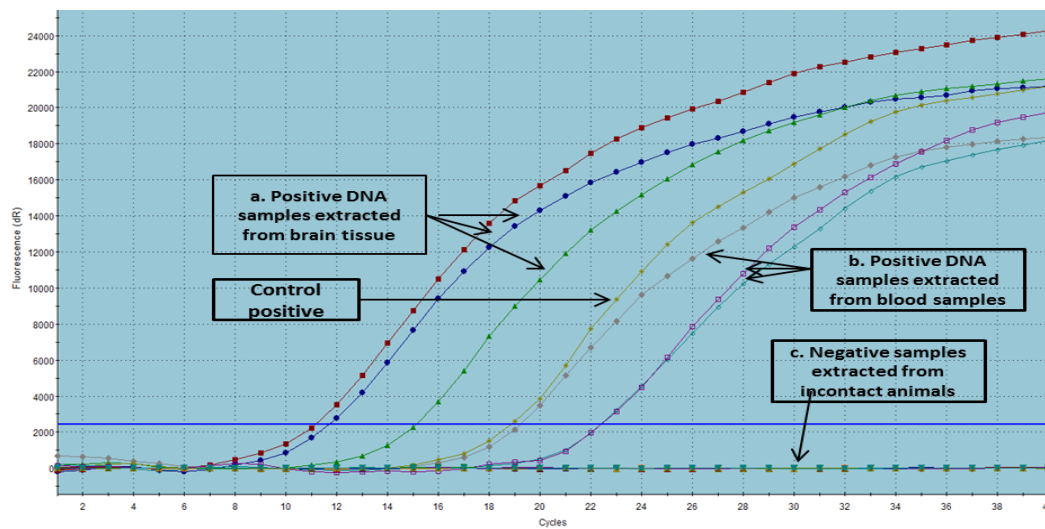


Figure (2). Results of real-time PCR showed positive Ct-values with three blood and brain samples of clinical infected sheep.

Discussion

Listeriosis caused by *L. monocytogenes* is an infectious disease affecting a wide range of mammalian species, including ruminants, monogastric animals and humans. In ruminants, among which sheep are the ones more commonly affected (Brugere-Picouxa, 2008). While, the majority of infections are subclinical, listeriosis in animals can occur sporadically or as epidemics (Kimberling, 1998). Several factors, including sudden changes in ration, climatic changes (e.g., extremely cold weather) and prolonged periods of transportation have been associated with the onset of clinical listeriosis in ruminants (Audurier *et al.*, 1980; Wagner *et al.*, 2005). Silage feeding is also considered to have some intrinsic immunosuppressive effect in sheep (Audurier *et al.*, 1980; Wagner *et al.*, 2005). 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.

The development of a molecular test for detection of *L. monocytogenes* in clinical samples was motivated by the need for a rapid and reliable test for the diagnosis of CNS listeriosis complementary to the culture. Indeed, the rapid

detection and identification of causative pathogens in bacterial meningitis are critical for a rapid adaptation of antibiotic treatment (Chiba *et al.*, 2009). Moreover, this test should be highly sensitive because CNS listeriosis often linked to the presence of low number of bacteria in CSF. The *hly* gene, part of the genome of *L. monocytogenes*, was chosen as a target for detection and quantification by real-time PCR of the *L. monocytogenes* infection, The *hly* gene is a major and specific virulent factor for *L. monocytogenes* which is necessary for the invasiveness of the bacteria (Kayal and Charbit, 2006).

In this study, the presence of *L. monocytogenes* infection was investigated in symptomatic sheep flocks. Our PCR analysis revealed the presence of *L. monocytogenes* in three brain and blood samples which were collected from clinically affected sheep while there were two brain samples positive with microbiological examination. In another hand, the PCR assay gave negative results with all samples collected from in contact animals. The specificity of the assay was validated in a previous study (Amagliani *et al.*, 2004). This molecular assay detected the infection successfully even when the cultural methods failed (Amagliani *et al.*, 2006). The negative results obtained in the symptomatic sheep with microbiological assay

could be justified by either a very small contamination rate or the absence of the living bacteria, due to the administration of antibiotics.

With respect to the conventional procedures, the two molecular methods presented a significantly shorter analysis time and possibly a higher sensitivity.

In conclusion, although the isolation of *L. monocytogenes* remains an important criterion for the diagnosis of listeriosis, the 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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