A case report of sporadic ovine listerial meningoencephalitis in Kosovo

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Summary
In 2018, a case of neural disease suspected of listeriosis was reported in a flock of sheep in Kosovo with the death of ewes and 5 lambs. Samples from the brain of only three dead animals were subjected to histopathological and bacteriological analysis. MALDI-TOF MS was applied to confirm suspected *Listeria* spp. isolates from culture and multiplex PCR was applied for molecular serotyping. All isolates were tested for antimicrobial susceptibility by microdilution broth method. The histopathological analysis of the brain specimens showed typical changes for *Listeria monocytogenes*. *Listeria* spp. was isolated in brain samples from all three animals, and all the isolates were confirmed using MALDI-TOF MS and PCR down to the species level (*Listeria monocytogenes*). The molecular characterisation using multiplex PCR revealed all isolates as *Listeria monocytogenes* serotype 4b. All *L. monocytogenes* isolates were found to be susceptible to penicillin, erythromycin, tetracycline, streptomycin, trimethoprim/sulfamethosazole, quinupristin/dalfopristin, kanamycin, vancomycin, and gentamicin but resistant to nitrofurantoin and lincomycin. This study shows the emergence of a highly virulent strain in sheep farms in Kosovo and a possible threat to public health.

Introduction

Listeriosis is an infectious disease caused by *Listeria monocytogenes*, a Gram-positive, intracellular, nonsporulating rod, which affects a wide range of animals as well as humans (George 2002, Nash *et al.* 1995). In both humans and livestock (sheep, cattle, goats, and less frequently, poultry), listeriosis can manifest as encephalitis, septicaemia, and abortion (Bartt 2000, Kumar *et al.* 2007, Brugere-Picoux 2008). The disease usually occurs sporadically, thus outbreaks are only occasionally reported (Walland *et al.* 2015). In animals, listeriosis outbreaks are primarily described in small ruminants (goats, sheep) and cattle (Wagner *et al.* 2005, Budrant *et al.* 2011, Dreyer *et al.* 2015). The neurological form, otherwise known as circling disease, is usually observed in farm animals where the bacterium gains entrance to the body through abrasions of the buccal mucosa. It ascends unilaterally along the trigeminal nerve, resulting in encephalitis. *L. monocytogenes* can spread intraaxonally within the brain along interneuronal connections both in retro- and anteretrograde directions (Oevermann *et al.* 2010, Granier *et al.* 2011, Henke *et al.* 2015). Listeriosis associated with encephalitis is more prevalent among ruminants, especially adult sheep, with an attack rate of 10-20% and a mortality rate of 5-10%, respectively. Lambs as young as 5 weeks old may develop the septicaemic form of the disease, whereas older feedlot lambs (4-8 months old) develop the encephalitic form (Wesley *et al.* 2002, Lozano *et al.* 2011).

Ruminants affected by listerial encephalitis generally show marked neurological symptoms, including ataxia, masticatory problems, failure of jaw closure, drooping of ears, upper eye lids and lips, swallowing problems, tongue palsy, circling, head tilt and leaning to one side, drooling of saliva, and facial paralysis (Braun *et al.* 2002, Kumar *et al.* 2007, Dons *et al.* 2007, Brugere-Picoux 2008, Rocha *et al.* 2013). Once *L. monocytogenes* enters the brain in small ruminants, its elimination is

less likely in comparison to cattle (Godreuil *et al.* 2003, Rocha *et al.* 2013). Sporadic ovine listeriosis cases are mainly attributted to serogroup 1/2a, 1/2b, and 4b (Low *et al.* 1993, Kotzamanidis *et al.* 2019). *L. monocytogenes* is typically found in soil, vegetables, plants, feces, sewage, genital secretions and the nasal mucous membranes of healthy animals. The disease occurs more frequently in the winter and the early spring and is associated with encephalitis after silage intake (Wiedmann *et al.* 1994, Bertsch *et al.* 2013). In these cases, mortality rates of 3.1% and 1.3% have been observed in ewes and lambs, respectively (Nash *et al.* 1995, Lyon *et al.* 2008). *L. monocytogenes* is commonly present in silage, but it can multiply only in silage which has not been fermented properly (pH 5.0-5.5) (Doumith *et al.* 2004, Kumar *et al.* 2007). Livestock intended for food production play an important role in the zoonotic transmission of the disease. Humans commonly acquire *L. monocytogenes* following consumption of products such as contaminated raw milk and, minimally processed foods of plant origin (contamination likely traced back to animal operations); however, rare cases of direct transmission have been reported (Heger *et al.* 1997, Nightingale *et al.* 2004). Outbreaks of human listeriosis have been mainly linked to consumption of ready-to-eat (RTE) foods, including soft cheeses. It should be noted that outbreak investigations and source attribution are often complicated by the typically long incubation time of *L. monocytogenes* in conjuction with the ubiquity of this pathogen, which often leads to contamination of foods not likely to be associated with listerial presence (Granier *et al.* 2011, Amato *et al.* 2017, Buchanan *et al.* 2017). Further, the occurrence of antibiotic resistance of isolates of *L. monocytogenes* from non-human samples has been reported for a number of important antibiotics, including tetracycline, doxycyline, erythromycin and sulfamethoxazole/trimethoprim (Vela *et al.* 2001, Granier *et al.* 2011, WHO 2016, Amato *et al.* 2017). Well-established breakpoints for use in determination of antimicrobial susceptibility of *L. monocytogenes* veterinary clinical isolates are not available, except for select antibiotics such as penicillin G and sulfamethoxazole/trimethoprim, complicating interpretation of antimicrobial susceptibility data and requiring extrapolation based on available information from other closely-related bacteria.

The aim of the current study was to provide descriptive analysis of an outbreak in sheep associated with high mortality, determine the cause of fatalities, identify and characterize the etiological agent, and determine the antibiotic susceptibility of the isolates.

Case report

Case presentation

In March 2018, 10 adult ewes as well as 8 lambs from a flock of 277 sheep in central Kosovo displayed symptoms such as depression and anorexia followed by neurological signs. Forty-eight hours from the initial onset of the symptoms, the affected ewes and lambs manifested severe clinical signs such as temperature of 40.5 °C, grinding of teeth (crunch empty), circling and tilting of the head, unilateral facial paralysis, drooping ears and lips and supporting the head against the manger and walls. Within 7 days after the disease onset, 6 adult sheep and 5 lambs died. Samples were obtained from the brainstem of the dead animals. A silage diet had been administered to the flock of sheep. Based on the narrative provided by the sheep flock owner, one of the dead lambs (3 month old), was exclusively fed with milk from an ewe.

Histopathological analysis and findings

In order to investigate the cause of the disease and the fatalities, samples from three dead animals (ewes and lamb) were obtained from the brainstem to conduct histopathological analysis. The samples were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin, 5 µm sections were stained with hematoxylin and eosin (H&E), and observed with a light microscope with 10x-40x objectives. Examination of the medulla oblongata revealed suppurative encephalitis characterized by microabscesses as well as neuroparenchymal lesions (Figure 2 and 3, black arrow) in all affected animals. Perivascular cuffing of lymphomononuclear cells was observed in the medulla oblongata with enhanced Virchow-Robin spaces (Figure 1, black arrow).

Isolation of *Listeria monocytogenes* from brain samples

At necropsy of two dead ewes and one lamb, two brain samples from each animal were collected for bacteriological analysis. Subsequently, samples were cultured in Fraser broth (Merck) and incubated aerobically for 24-48 h at 37 °C. After incubation, an aliquot was streaked onto PALCAM Listeria Selective Agar (Merck) and incubated aerobically for 24-48 h at 37 °C. Presumptive identification of *L. monocytogenes* was based on the observation of typical colony morphologies on PALCAM agar, Gram staining, haemolysis patterns, catalase and oxidase reactions, and tumbling motility. The characteristic Gram-positive coccobacilli (short rods)

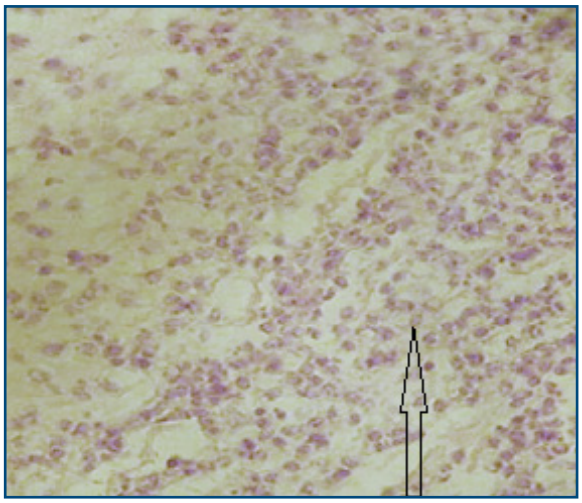

Figure 1. *Neuroparenchymal lesions from infiltration of lympho mononuclear cells (Hematoxylin and Eosin stain; 40× Objec.).*

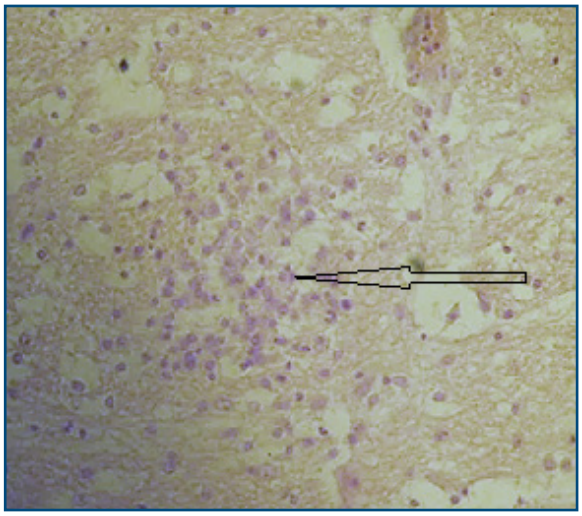

Figure 2. *Microabscess in medulla oblongata (Hematoxylin and Eosin stain; 40× Objec.)*

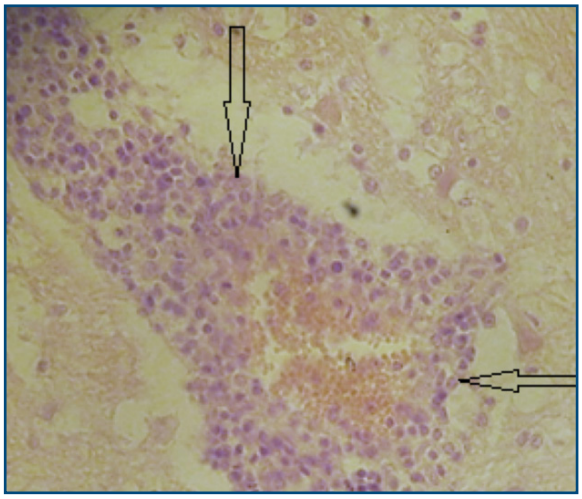

Figure 3. *Perivascular infiltration of lympho mononuclear cells in medulla oblongata (Hematoxylin and Eosin stain; 40× Objec.)*

which were catalase positive and oxidase negative, and displayed umbrella shaped growth pattern in motility medium as well as tumbling motility in wet mounts at room temperature were considered as 'presumptive' *Listeria* isolates. Typical *Listeria* colonies were transferred to cryotubes containing Brain Heart Infusion Broth (BD Difco) with 20% glycerol and maintained frozen at - 20 °C until used for confirmation.

Confirmation of isolates of *L. monocytogenes*

Downstream confirmation analyses revealed the presence of *L. monocytogenes* in six brain samples (three different animals) analyzed (Table I). The confirmation of isolates of *L. monocytogenes* was achieved by matrix-assisted laser desorption/ ionization mass spectrometry (MALDI-TOF MS)-based identification. MALDI-TOF MS and biotyping was performed using the BrukerUltraflex II TOF/TOF in positive ion reflector mode pre-calibrated with bacterial test standard (Bruker) according to the manufacturer's instructions using an ethanol/ formic acid extraction protocol. Spectral analyses and sample identifications was performed using the BrukerBiotyper software (Ver. 3.1.). Identifications were only accepted if scored ≥ 2.0 by the Biotyper algorithm. All isolates were confidently identified by MALDI-TOF MS as *L. monocytogenes* (score higher than 2.4).

Molecular serotyping of *L. monocytogenes*

L. monocytogenes strains previously confirmed by MALDI-TOF MS, including five isolates (B1, B2, B3, B4, and B5) from the first sheep, five isolates (B6, B7, B8, B9, and B10) from the second sheep, and eight isolates (B 11, B12, B13, B14, B15, B16, B17, and B18) from the lamb, were subjected to molecular serotyping by targeting six genes of *L. monocytogenes*, *lmo0737* (691 bp), *lmo1118* (906 bp), *ORF2819* (471 bp), *ORF2110* (597 bp), *prfA* (274 bp), and *prs* (370 bp) (Doumith *et al.* 2004, Kerouanton *et al.* 2010). PCR was performed

Table I. *Number of samples and* L. monocytogenes *isolates included in the study.*

Number of samples (brain)	Strains*	Serotypes
2 (from sheep 1)	B1, B2, B3, B4, B5	4b
2 (from sheep 2)	B6, B7, B8, B9, B10	4b
2 (from lamb 1)	B11, B12, B13, B14, B15, B16, B17, B18	4b

*B = Clinical isolates from the brain of sheep and lamb affected of meningoencephalitis.

with 2 separate groups, one for genes *prfA* and *prs*, and the other four genes *lmo0737, lmo1118, ORF2819*, and *ORF2110*. The molecular serotyping using multiplex PCR revealed *prfA, prs, ORF2819*, and *ORF2110* genes from all isolates, indicating that the isolates belonged to *L. monocytogenes* serotype 4b.

Antimicrobial susceptibility testing of *L. monocytogenes* isolates

All strains were tested for antimicrobial susceptibility by broth microdilution using the Sensititre® GPN3F dehydrated panel (Trek Diagnostic Systems, Cleveland, OH) following the manufacturer's instructions. The following antimicrobial agents were used for the *in vitro* microdilution broth testing protocol: penicillin, erythromycin, sulfamethoxazole/trimethoprim, chloramphenicol, tetracycline, daptomycin, streptomycin, tylosin tartrate, quinupristin/dalfopristin, linezolid, nitrofurantoin, kanamycin, ciprofloxacin, vancomycin, lincomycin and gentamicin. The breakpoints used for the determination of the minimum inhibitory concentrations (MIC) using the broth microdilution method, were those recommended by Clinical & Laboratory Standards Institute (CLSI 2014) for closely-related bacteria (*Staphylococcus* spp., *Enterococcus* spp., *Streptococcus* spp.). There was one exception to this pattern, namely for penicillin for which specific *Listeria* breakpoints are defined. *Staphylococcus aureus* ATCC 29213 was used as quality control strain, with susceptibility testing results in the expected range.

All 18 *L. monocytogenes* strains tested were found to be susceptible to penicillin (MIC 0.5-1 µg/mL), erythromycin (MIC 0.25 µg/mL), tetracycline (MIC 1 µg/mL), ciprofloxacin (MIC 1 µg/mL), tylosin tartrate, sulfamethoxazole/trimethoprim, streptomycin, vancomycin, and gentamicin, but

Table II. *MICs of 16 antimicrobial agents for* L. monocytogenes *isolates from ovine meningoencephalitis.*

Antimicrobials	Number of isolates (n = 18) with MIC of (mg/l)															
	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024
Penicillin	-	-	-	2 (11.1)	14 (77.8)	2 (11.1)	-	-	-	-	-	-	-	-	-	-
Erythromycin	-	-	-	18 (100)	-	-	-	-	-	-	-	-	-	-	-	-
Tetracycline	-	-	-	-	-	18 (100)	-	-	-	-	-	-	-	-	-	-
Ciprofloxacin	-	-	-	-	-	18 (100)	-	-	-	-	-	-	-	-	-	-
Chloramphenicol	-	-	-	-	-	-	-	-	15 (83.3)	2 (11.1)	1 (5.6)	-	-	-	-	-
Sulfamethoxazole/ Trimethoprim	-	-	-	-	18 (100)	-	-	-	-	-	-	-	-	-	-	-
Daptomycin	-	-	-	-	-	-	11 (61.1)	7 (38.9)	-	-	-	-	-	-	-	-
Vancomycin	-	-	-	2 (11.1)	-	14 (77.8)	2 (11.1)	-	-	-	-	-	-	-	-	-
Streptomycin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	18 (100)	-
Nitrofurantoin	-	-	-	-	-	-	-	-	-	-	-	18 (100)	-	-	-	-
Tylosin tartrate	-	-	-	-	-	-	-	16 (88.9)	2 (11.1)	-	-	-	-	-	-	-
Gentamicin	-	-	-	-	-	-	-	-	-	-	-	-	18 (100)	-	-	-
Quinupristin/ dalfopristin	-	-	-	-	12 (66.6)	5 (27.8)	1 (5.6)	-	-	-	-	-	-	-	-	-
Lincomycin	-	-	-	-	-	-	-	-	18 (100)	-	-	-	-	-	-	-
Linezolid	-	-	-	-	1 (5.6)	-	2 (11.1)	15 (83.3)	-	-	-	-	-	-	-	-
Kanamycin	-	-	-	-	-	-	-	-	-	-	-	-	17 (94.4)	-	-	1 (5.6)

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resistant to lincomycin with an observed MIC of 8 µg/mL, daptomycin (MIC 2-4 µ/mL) and intermediate to nitrofurantoin with an observed MIC of 64 µg/mL. Furthermore, a number of isolates were classified as sensitive to some other antimicrobials such as kanamycin (17/18), quinupristin/dalfopristin (17/18), chloramphenicol (15/18), and linezolid (3/18); resistant to chloramphenicol (1/18) and kanamycin (1/18); and intermediate to linezolid (15/18), chloramphenicol (2/18), and quinupristin/dalfopristin (1/18) (Table II).

Discussion

The findings of suspected neurological disease presented in this case study were established through histopathology and horizontal isolation of the etiological agent, *L. monocytogenes* from brain tissues. Histopathological findings observed within the brainstem of these animals are consistent with those previously described for ruminant encephalitic listeriosis (Maxie and Youssef 2007, Zachary 2012). The finding of a seasonal occurrence of ruminant listeriosis in this study correlates well with the findings reported by Wiedmann and colleagues (Wiedmann *et al.* 1994). That specific study reported isolation of *Listeria* from brains of sheep and goats affected with listerial encephalitis and from silage consumed by the animals in the winter and early spring. We found that only one serotype, serotype 4b, was implicated as the etiological agent in this outbreak. In another similar study conducted in Greece, serotype 4b was reported as the predominant strain (81%) isolated in small animals, in which the neural form of the disease was diagnosed (Giannati-Stefanou *et al.* 2006).

The antimicrobial susceptibility testing results in our study overwhelmingly support the presence of highly susceptibile *L. monocytogenes* isolates (n = 18). We report the following levels of susceptibility for the isolates tested: penicillin (100%), erythromycin (100%), tetracycline (100%), ciprofloxacin (100%), tylosin tartrate (100%), sulfamethoxazole/trimethoprim (100%), vancomycin (88.9%), streptomycin (100%), gentamicin (100%), kanamycin (94.4%), quinupristin/dalfopristin (94.4%), and chloramphenicol (83.3%). All isolates (100%) were resistant to lincomycin and daptomycin. Resistance was also observed to chloramphenicol and kanamycin (5.6%). On the other hand, our study indicated that the following proportions of *L. monocytogenes* isolates could be included in the intermediate category: nitrofurantoin (100%), linezolid (83.3%), chloramphenicol (11.1%), and quinupristin/dalfopristin (5.6%). The MIC ranges obtained in this study for penicillin, erythromycin, gentamicin, and vancomycin, are quite similar to those reported for strains of *L. monocytogenes* from ovine meningoencephalitis in a study by Vela and colleagues (Vela *et al.* 2001). While in other authors' studies *L. monocytogenes* isolates have shown resistance to tetracycline (Vela *et al.* 2001, Counter *et al.* 2007), the isolates in the current study were susceptible to this antimicrobial. Based on the *in vitro* analysis, it would be expected that penicillin would provide an effective treatment during administration of antibiotic therapy in the current clinical settings. Furthermore, low-level resistance to a fluoroquinolone such as ciprofloxacin has been associated with the presence of efflux pumps and potentially the use of fluoroquinolones in animal production, especially broiler production (Godreuil *et al.* 2003). It is not unusual that all isolates displayed resistance to daptomycin, which corroborated findings of other studies which do not recommend the use of daptomycin for treatment of listeriosis cases (Noll *et al.* 2018, Spanjaard and Vandenbroucke-Grauls 2008). Multiple mechanisms, including some that are transferable, have been reported to be involved in conferring resistance to lincosamides in *Listeria* spp. (Bertsch *et al.* 2013). Different *L. monocytogenes* isolates originating from the same tissues (i.e. brain) were shown to contain different antimicrobial resistance phenotypes. This could also indicate further differences between the different strains of isolates of *L. monocytogenes* 4b, including other virulence factors. Furthermore, this study demonstrates the presence of highly pathogenic *L. monocytogenes* serotype 4b strains causing a neural form of listeriosis in small ruminants in Kosovo, which is being reported for the first time.

Listeriosis-positive sheep flocks may pose a grave public health risk, as a great number of popular food products derived from these animals are often consumed in the region, greatly increasing opportunities for zoonotic transmission. Therefore, in such cases preventive measures have to be undertaken to avoid the transfer of *L. monocytogenes* to processed milk and other dairy products, although human listeriosis cases are yet to be reported in Kosovo.

Lastly, the results of our study indicate that *L. monocytogenes* from ovine listeriosis manifests susceptibility to most commonly utilized antimicrobials; however, several instances of intermediate susceptibility and full resistance identified in the tested isolates suggests a future need for close monitoring and surveillance of antimicrobial resistance in *L. monocytogenes* strains of animal origin in Kosovo.

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