

# Detection of *Listeria monocytogenes* in cheese and in cow mastitis – a case report

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## Abstract

*Listeria monocytogenes* is ubiquitous and causes listeriosis, which manifests in animals in the form of septicaemia, encephalitis, abortions, and, rarely, mastitis. In humans, food is the main source of infection, which is the reason food business operators take samples and test for *Listeria*. We report a case of *L. monocytogenes* detection in traditional cheese made from raw milk, and subsequent investigation revealed that *L. monocytogenes* was excreted

from a cow mammary gland. Although treatment was carried out according to the results of the antibiogram, there was no improvement in the subclinical mastitis and the excretion of *L. monocytogenes* after treatment was  $8.8 \times 10^2$  cfu/mL, and therefore the cow was removed from the herd.

**Key words:** *Listeria*; mastitis; cheese; bovine; milk; sampling

## Introduction

*Listeria monocytogenes* is a gram positive, rod-shaped bacterial species. It is facultatively anaerobic, motile, catalase-positive, oxidase-negative and expresses a beta-haemolysin which, in contrast to apathogenic *Listeria* species, causes beta-haemolysis on blood agar. It is classified in 14 serovars based on the somatic and flagellar antigens, of which serovars 4b, 1/2a, 1/2b and 3 are the most widespread. The pathogen is ubiquitous: it is found in soil, water and on farms (Papić et al., 2019), as well as on the surface of plants.

It has been isolated from 42 mammal species and many bird species, including

seagulls (Humski et al., 2022). It causes listeriosis, which manifests in animals as septicaemia, encephalitis, abortions and, more rarely, mastitis and conjunctivitis. Human infections occur after consumption of contaminated food rather than via contact with infected animals. Children, the elderly, pregnant women and immunocompromised patients are susceptible to listeriosis (Swaminathan et al., 2007). Listeriosis occurs in two forms: the first with mild symptoms, that may or may not include gastroenteritis, and the second in a more severe form with meningitis, septicaemia and a high mortality rate. Pregnant

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women can abort the foetus or give birth to an infected child.

As an intracellular pathogen, *Listeria monocytogenes* most frequently causes encephalitis or meningoenkephalitis and uterine infections in adult ruminants. The encephalitic form of listeriosis in animals is characterised by neurological symptoms, including circling movements, excessive salivation and unilateral facial paralysis. In cattle, the disease lasts one to two weeks, in calves and sheep it is shorter, 2-4 days, and is fatal. Infections of the uterus are characterised by late abortions or septicaemia in newborns. Abortions in sheep and goats appear epizootic, while in cattle they occur sporadically (Cvetnić, 2013).

In rare cases, mastitis may occur. In 1980, a case of bovine mastitis caused by *L. monocytogenes* serotype 4 was described (Gitter et al., 1980), while Fedio et al. (1990) described a case of subclinical mastitis caused by *L. monocytogenes* serotype 1 in an udder quarter. Persistent excretion of *L. monocytogenes* in milk from cows and sheep suffering from subclinical mastitis has been confirmed (Winter et al., 2004), so subclinical *Listeria* mastitis should not be neglected as a potential source of milk contamination (Papić et al., 2019).

In studies conducted in Croatia, *L. monocytogenes* was isolated from 4.27% of cake samples (Uhitil et al., 2004), from 13.39% of authentic products made from raw milk (Kožačinski and Hadžiosmanović, 2001) and from 21.4% of raw and frozen poultry meat samples (Humski et al., 2001). The prevalence of this bacterium was investigated in other samples of animal origin (faeces and organs of cattle, pigs, goats, poultry, pets, wild animals), where *L. monocytogenes* was detected by cultural and immunoenzymatic methods in 0.4% and 0.5% of 4441 examined samples (Humski et al., 1999).

In analysis of fresh cheese samples from the environs of the City of Zagreb, a relatively low frequency of this bacterial species was found in these products. Markov et al. (2009) used the polymerase chain reaction method to detect the presence of *L. monocytogenes* and found *Listeria* in one of 60 analysed samples of homemade fresh cheese from this area. Dobranić et al. (2010) did not detect this bacterial species in their study on the hygiene of cottage cheese from Zagreb markets, while Humski et al. (2011), when analysing cottage cheese using cultural and immunoenzymatic methods, detected *Listeria monocytogenes* in one of 30 samples tested (3.3%), and the species *L. innocua* in three samples (10%).

In Croatia, there are many traditional cheese types made from cow, sheep and goat milk (Ivček, 2021). This particular type of cheese is made from raw cow milk obtained from two consecutive milkings: evening and morning. The mixed milk is heated to 30–35°C, rennet is added and curdling takes 30 to 50 minutes. The curd is broken by hand or with a wooden spoon into pieces 2–3 cm in diameter. The curd rests for one hour at 40°C. It is then shaped and salted by hand. The shaped cheese is smoked for 2 to 3 days and finally matured for 4 weeks (Lukač Havranek, 1995; Barukčić and Tudor Kalit, 2019).

The aim of this article was to describe the actions taken to detect the primary source of *L. monocytogenes* detected in the cheese.

## Materials and methods

The detection of *L. monocytogenes* was performed according to HRN EN ISO 11290-1:2017 using a *Listeria* Fraser Broth Base Half Concentration with *Listeria* Fraser Supplement (Biolife Italiana S.r.l, Italy) for primary enrichment, and

a *Listeria* Fraser Broth Base with *Listeria* Fraser Supplement (Biolife Italiana S.r.l, Italy) for secondary enrichment. After incubation of the primary and secondary enrichment media, two different agar plates were inoculated by a loop: ALOA and PALCAM (Biolife Italiana S.r.l, Italy).

*L. monocytogenes* was enumerated according to HRN EN ISO 11290-2:2017 by inoculation of 1 mL initial suspension and decimal dilutions on the surface of ALOA (Biolife Italiana S.r.l, Italy) in a 140 mm Petri dish.

*L. monocytogenes* was confirmed by further testing of the suspected colonies. Blood agar plates, prepared from Blood Agar Base (Biolife Italiana S.r.l, Italy) with sheep blood additive (BIOSAP SO, Biognost d.o.o., Croatia) were inoculated and incubated at 37°C for 24 hours to investigate haemolysis. API *Listeria* (bioMérieux SA, France) was used for the utilization of L-rhamnose and D-xylose, and for other tests.

Environmental swabs were collected using the moistened sponge/cloth technique described in HRN EN ISO 18593:2019. The sponge was moistened with 10 mL Maximum Recovery Diluent (Biolife Italiana S.r.l, Italy) and transported in a sterile plastic bag. The selected surfaces were sampled by wiping horizontally and vertically with even and firm pressure, alternating the side of the sponge and ensuring that the entire area was sampled. The sponge was placed back into the plastic bag and kept moist and cool until analysis. The sponge was analysed for the detection of *L. monocytogenes* according to HRN EN ISO 11290-2:2017, treated as a 10 g sample.

Antimicrobial susceptibility was determined using the EUCAST disk diffusion method (Anon., 2022). This method uses antimicrobial disks with Mueller Hinton Agar II (Biolife Italiana S.r.l, Ita-

ly). The bacterial culture was prepared by suspension in saline solution to the density of a 0.5 McFarland turbidity standard. The suspension was applied to a surface of Mueller Hinton Agar II with a cotton swab, and then the antimicrobial discs were lightly pressed onto the agar. The plate was incubated at 35±1°C for 18±2 hours. The diameter of the zone of inhibition was measured with callipers.

## Case presentation

A food business operator (FBO), a cheese manufacturer, took a sample of a semi-hard cheese made from raw milk, and delivered it to the laboratory by a technician in a refrigerated vehicle, as part of its auto-control system. The sample consisted of five units. Sampling was carried out in accordance with the FBO's self-monitoring programme.

The analysis yielded the following results:

Parameter	<i>L. monocytogenes</i> /25 g
Unit	
Unit 1	present
Unit 2	present
Unit 3	present
Unit 4	present
Unit 5	present

The results were immediately communicated to the FBO and new sampling was organised immediately. The FBO collects milk from several suppliers and at the time of sampling, milk originated from two suppliers: supplier "A" and supplier "B", both of which were sampled. Samples were also taken from cheese rennet and three batches of semi-hard cheese made from raw milk. All samples were analysed for the presence

of *L. monocytogenes* in 25 g or 25 mL. *L. monocytogenes* was detected in the raw milk sample "B", but not in sample "A" or any of the three cheese samples. In a discussion with the managing director of the FBO, it was found that supplier "B" was a small dairy farm with six lactating cows. On the next visit to the FBO facilities, a batch of cheese and five surface swabs were taken with a moistened sponge: two workbenches, a cheese press, a cheese mould and a milk bucket.

On the same day, supplier "B", a dairy farm, was visited and samples of bulk milk from milk refrigerators, a rinse from a milking machine and haylage were taken. In discussion with the farm manager, it was noticed that a particular cow (M) had previously been treated for mastitis and fibrosis was palpated in a quarter of the udder during clinical examination. Otherwise, cow M appeared to be healthy. A milk sample was taken

from cow M based on the anamnesis and clinical examination. Again, all samples were tested for the presence of *L. monocytogenes*, with the following result: *L. monocytogenes* was present in the bulk milk and in the sample from cow M and was not detected in the surface swabs, cheese, haylage or the milking machine rinse.

Further samples were then taken at the dairy farm: two surface swabs, bulk milk, four samples from cow M (one from each udder quarter) and samples from other cows on the farm: R, S, M2 and K, with the following results in Table 1.

The bacterial culture isolated from cow M was tested for antibiotic sensitivity and showed sensitivity to kanamycin, gentamicin, imipenem and neomycin.

Treatment of the udder was carried out according to the results of the antibiogram and then tested for the presence and enumeration of *L. monocytogenes*. Af-

Table 1. Result

	Bucket	Lid	Front left	Front right	Rear left	Rear right
Surface	Detected	Detected				
Cow M			Absent	Absent	Absent	Detected
Cow R			Absent	Absent	Absent	Absent
Cow S			Absent	Absent	Absent	Absent
Cow M2			Absent	Absent	Absent	Absent
Cow K			Absent	Absent	Absent	Absent
Bulk milk						Detected 120 cfu/mL

Table 2. Result

Mammary gland	Detection of LM	Enumeration of LM
Front left	Absent	<10
Front right	Absent	<10
Rear left	Absent	<10
Rear right	Present	8.8 x 10 <sup>2</sup>

ter treatment, the results were as follows in Table 2.

As neither the subclinical mastitis nor the excretion of *L. monocytogenes* improved, cow M was culled.

## Discussion

*L. monocytogenes* was detected in cheese sampled as part of the FBO self-monitoring programme. The source of contamination was identified through timely reporting of results and more detailed sampling at the FBO facility and premises of supplier "B".

The results of sampling at farm "B" proved that *L. monocytogenes* was present in bulk milk and that cow M was excreting *L. monocytogenes*. The results also showed good hygiene practices on the farm as *L. monocytogenes* was not detected in milking machines and other equipment. It was also evident that *L. monocytogenes* was no longer present in the FBO after supplier "B" had been excluded from production. A sample of haylage and from other cows showed that the feed was not the source of infection and that other cows were healthy and did not excrete *L. monocytogenes*, meaning that cow M's milk, more specifically milk from the right rear quarter, contaminated the contents of the milk refrigerator. As the chronic subclinical mastitis caused by *L. monocytogenes* did not respond to treatment, the cow in question was culled.

Mastitis caused by *L. monocytogenes* is relatively rare, but does occur. Jensen et al. (1996) examined samples from 1,132,958 cows from 36,199 herds over a period of 23 years (1972 to 1994). The percentage of cows infected with *L. monocytogenes* varied between 0.01 and 0.1% (mean 0.04%) and the percentage of herds with an infected cow varied between 0.2 and 4.2% (mean 1.2%), indicating a low

but constant level of infection. In another study, *Listeria* spp. were isolated from 12 of 725 (1.66%) samples from cows that had previously tested positive for mastitis using the California mastitis test and somatic cell count. Of these 12 samples, 4 (0.55%) were confirmed as *L. monocytogenes* (Rawik et al., 2007). *Listeria* spp. were detected in 21 of 207 bovine mastitis milk samples from dairy farms in Iran, including *L. monocytogenes* ( $n=17$ ), *L. innocua* ( $n=3$ ) and *L. ivanovii* ( $n=1$ ) (Jamali and Radmehr, 2013).

*L. monocytogenes* mastitis has also been detected in other animal species such as sheep, buffaloes and goats. In India, a total of three isolates of *L. monocytogenes* were recovered from 85 mastitic milk samples (47 buffaloes and 38 cows). All three isolates of *L. monocytogenes* were serotype 4b (Mahendra et al., 2010). In 1998, the prevalence of subclinical infection of the mammary gland with *L. monocytogenes* in ewes was 3.1% at the first and second sampling and 6.2% at the third sampling. Four ewes with a subclinical infection of the mammary gland with *L. monocytogenes* were euthanised (Fthenakis et al., 2014). In a study on goat and sheep milk, pathogenic *Listeria* spp. were found in 5.6% of goat and 3.9% of sheep milk samples, with 33.3% and 25% of these selected samples containing *L. monocytogenes*, respectively (Osman et al., 2014). In another study, Osman et al. (2014) detected *Listeria* species in camel milk: *Listeria* spp. were isolated from 4% of samples, with 1.0% confirmed as *L. monocytogenes*.

Even smaller amounts of colony-forming units per volume can pose a risk of infection, as *L. monocytogenes* grow in milk (Boljkovac, 2012) and cheese (Schvartzman et al., 2010; Lobacz et al., 2013; Dodić, 2017). It is clear that animals can excrete *L. monocytogenes* in different concentrations. A case was reported of a

clinically healthy goat that excreted  $>2000$  cfu/mL *L. monocytogenes* in its milk and contaminated bulk milk. After the goat was culled from the herd, the bulk milk tested negative (Addis et al., 2019). In a case of subclinical mastitis in a cow studied for two months, excretion was found to be  $4 \times 10^4$  cfu/mL (Wagner et al., 2019), while a sheep excreted  $9 \times 10^1$  to  $2.95 \times 10^5$  cfu/mL, the milk was contaminated with  $5.7 \times 10^3$  cfu/mL and the cheese product with  $2.0 \times 10^2$  cfu/mL (Schoder et al., 2003). One of 180 negative cows had a shedding of 280 cfu/mL, similar to our case, which was  $8.8 \times 10^2$  cfu/mL after antibiotic treatment. As in our case, the infection was resistant to antibiotic treatment (Hunt et al., 2012).

Although *L. monocytogenes* is not considered particularly udder invasive (Fedio et al. 1990; Cvetnić, 2002), in experimental infections of the mammary glands of cows in 1995, inoculated quarters developed chronic subclinical mastitis with occasional clinical episodes. The results were similar to those of natural *Listeria* mastitis. Only one of four quarters was cured after treatment only on "drying off". *L. monocytogenes* was isolated from the supramammary lymph nodes (Bourry et al., 1995) and similar results were found by Tzora et al. (1998) in ewes after inoculation of *L. monocytogenes* into the mammary gland.

A reliable microbiological test with the determination of sensitivity to antimicrobial agents is the basis of mastitis control in addition to clinical examination (Sukalić et al., 2021), although in our case, as in other cases, antibiotic treatment showed no results.

Mastitis caused by *L. monocytogenes* is extremely rare (Turk et al., 2017), but represents a food safety factor, and to the authors' knowledge, this is the first case described in Croatia.

## Conclusion

Following the detection of *L. monocytogenes* in traditional cheese made from raw milk by extensive sampling in a cheese production plant and on a dairy farm, a cow excreting *L. monocytogenes* was identified. Antibiotic treatment was administered according to an antibioticogram, but the cow still excreted  $8.8 \times 10^2$  cfu/mL and was therefore removed from the herd. *L. monocytogenes* is a rare cause of mastitis, but a particularly important factor for food safety, as it multiplies in milk and cheese at low temperatures. Excreted *L. monocytogenes* contaminates the bulk milk, which apart from health risks can also lead to economic damages. The excretion of *L. monocytogenes* from the mammary gland can be a potential cause of infection in other animals and, due to resistance to antibiotic therapy, the loss of infected cows can lead to economic losses. Milk producers and processors should therefore regularly test milk for *L. monocytogenes*.

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## Nalaz *Listeria monocytogenes* u siru i mastitisu krave – prikaz slučaja

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*Listeria monocytogenes* je ubikvitarna i uzrokuje listeriozu koja se u životinja manifestira u obliku septikemije, encefalitisa, pobačaja i rijetko mastitisa. Za ljude je glavi izvor zaraze hrana, zbog čega subjekti u poslovanju s hranom uzimaju uzorke hrane i površina i testiraju na prisutnost bakterije *Listeria*. Izvješćujemo o slučaju *L. monocytogenes* otkrivene u tradicionalnom siru od sirovog mlijeka, čijim se

istraživanjem došlo do krave koja je izlučivala *L. monocytogenes* iz mliječne žlijezde. Iako je liječenje provedeno prema nalazima antibiograma, nije bilo poboljšanja subkliničkog mastitisa, a izlučivanje *L. monocytogenes* nakon liječenja iznosilo je  $8,8 \times 10^2$  cfu/mL, a krava je bila izlučena iz proizvodnje.

**Ključne riječi:** *Listeria*, mastitis, sir, govedo, mlijeko, uzorkovanje