

**ISOLATION OF *LISTERIA MONOCYTOGENES* IN NEURAL FORMS OF LISTERIOSIS AND ABORTIONS IN RUMINANTS**

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*L. monocytogenes is a food borne pathogen capable of causing serious invasive diseases in humans and animals, including abortion, septicemia, meningitis and meningoencephalitis. Isolation of the agent is the most accurate diagnostic method in all situations of suspected L. monocytogenes infection. Direct isolation of L. monocytogenes is relatively simple in cases when the number of organisms is very large, such as septicaemic disease forms. Isolation is quite difficult if the agent is present in very small quantities, such as in encephalitis, or if the sample is highly contaminated. In this paper we presented the isolation of L. monocytogenes from clinical samples by using selective media for enrichment and isolation, combined with the cold enrichment technique. In our investigation we isolated L. monocytogenes from 18 of the total of 46 investigated tissue samples originating from animals with clinical diagnosis of listeriosis. We also presented the basic differential-diagnostic procedure in relation to the mimicking bacterial species.*

*Key words: L. monocytogenes, isolation, diagnostic procedure*

INTRODUCTION

*L. monocytogenes is a food borne pathogen capable of causing serious invasive diseases in humans and animals, including abortion, septicemia, meningitis and meningoencephalitis (Low and Donachie, 1997). L. ivanovii causes the disease in animals only, mainly sheep. The pathogen is commonly found in soil and water and on plant material, particularly that undergoing decay, and these environments are regarded as the natural habitat of the organism (Rocourt and Seeliger, 1985). The term listeriosis encompasses a wide variety of disease symptoms; hence isolation of the agent is the most accurate diagnostic method in all situations of suspected L. monocytogenes infection.*

*Direct isolation of L. monocytogenes is relatively simple in cases when the number of organisms is very large, such as septicaemic disease forms. Isolation is quite difficult if the agent is present in very small quantities, such as in encephalitis, or if the sample is highly contaminated. The traditional procedure for the isolation of L. monocytogenes from animal tissues is the direct cultivation on*

blood agar or some other enriched medium and parallel cold enrichment technique including weekly subcultivation during 12 weeks (Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2004). One of the first reports on survival of *L. monocytogenes* at low temperatures is the paper of Biester and Schwarte published in 1939. The isolation technique, based on enrichment during several weeks at 4°C, was described some ten years later by Gray *et al.* (1948). Cold enrichment resulted in a larger number of positive samples in comparison to the incubation at higher temperatures. The main drawback of this method is the long incubation period (several weeks or months) making new isolation methods involving selective media for enrichment and isolation more suitable in daily routine practice.

After major listeriosis outbreaks in USA two federal agencies: United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) and Food and Drug Administration (FDA) of the Department of Health and Human Services (DHHS) (Hitchins, 1995) prescribed methods for the detection of *L. monocytogenes*. USDA method was suitable for meat products (red meat, poultry) and eggs, while FDA method was applicable for other food products, such as dairy products, seafood and vegetables. However, this is not clearly confirmed in several studies conducted to compare these two methods using the same samples (Beumer and Hazeleger, 2003). Some further conventionally applied methods are the AOAC and ISO 11290 standards.

The most commonly used selective agents added to enrichment media which are also recommended by the FDA, USDA and ISO, are acriflavine, nalidixic acid and cycloheximide. Acriflavine inhibits RNA synthesis and mitochondriogenesis. Ralovich *et al.* (1971) first described its application in the media for isolation of *Listeria spp.* The authors confirmed growth of *L. monocytogenes* in the presence of acriflavine at a concentration of 40 mg/L, which is sufficient to suppress the growth of Gram-positive cocci. Nalidixic acid inhibits the growth of Gram-negative micro-organisms and is often combined with other inhibition agents such as cycloheximide that inhibits protein synthesis in eucariotic cells by binding to 80S ribosomal RNA.

Many solid media have been developed for the isolation of *Listeria* from enrichment media or for direct plating methods. Until the 1990s all commercially available isolation media for *Listeria* suffered from the disadvantage that they offered no elective feature to distinguish *L. monocytogenes* from non-pathogenic listerias (Beumer and Hazeleger, 2003). Newly developed media enable the differentiation of *L. monocytogenes* from other *Listeria spp.* that is based on the production of a phosphatidylinositol-specific phospholipase C by *L. monocytogenes*, which hydrolyses a specific substrate added to the medium, producing an opaque halo around the colonies. One of such media is the Agar Listeria according to Ottoviani and Agosti (ALOA) (Ottaviani *et al.*, 1997).

The use of enrichment media for the isolation of *L. monocytogenes* from food is inevitable, which is due to the very small amount of bacteria in most food products (<10<sup>2</sup> cfu/g). For the same reason, these media are used also for clinical samples when direct isolation is unsuccessful. In this paper we presented the isolation of *L. monocytogenes* from clinical samples by using selective media for

enrichment and isolation, combined with the cold enrichment technique. We also presented the basic differential-diagnostic procedure in relation to the mimicking bacterial species.

#### MATERIAL AND METHODS

*Material.* In our investigation we used the following tissues:

- a. Tissue samples of the brain and medulla oblongata of sheep died with symptoms of CNS disease;
- b. Samples of visceral organs of lambs died of septicaemia;
- c. Aborted fetuses of cows and sheep, as well as placenta and amniotic fluid.

*Isolation media:*

- a. blood agar with 5% sheep blood;
- b. blood agar with addition of nalidixic acid;
- c. Listeria enrichment broth base (Biolife);
- d. Listeria acc. to Otaviani&Agosti (ALOA) agar (Biolife)
- e. Thioglycollate medium

*Bacteriology methods:*

Samples were processed immediately after admission to the laboratory using common bacteriology procedures, i.e. direct plating on blood agar with 5% sheep blood, blood agar with addition of nalidixic acid, MacConkey agar and thioglycollate medium. At the same time, all tissue samples were macerated in the grinder, homogenized with saline and inoculated into the liquid enrichment medium – Listeria enrichment broth base (Biolife). The liquid medium was incubated at 4°C and 30°C, and subcultures were made at 24 hour intervals on the selective solid medium – Listeria acc. to Otaviani & Agosti (ALOA) agar of the same manufacturer. All solid media were incubated aerobically at 37°C. After 24 hours of incubation at 37°C the thioglycollate medium was subcultured onto two blood agar plates. The first and the second plate were incubated in aerobic and anaerobic conditions, respectively.

*Identification of the agent:*

*Listeria*-suspected colonies were cultivated in pure culture on blood and neutral agar prior to be subjected to tinctorial (Gram stain) analysis and catalase and oxidase tests. Ability of producing the CAMP phenomenon was investigated using double-CAMP test on blood agar. On the two parallel vertical lines strains of *Staphylococcus aureus* and *Rhodococcus equi* were inoculated, while control strains *Streptococcus agalactiae* and *Listeria ivanovii* (positive control) and the investigated isolates were inoculated horizontally.

*Biochemical properties* of the isolate were examined using the following line: aesculin, glucose, lactose, saccharose, maltose, arabinose, xylose, adonit, sorbitol, mannitol, dulcitol, inulin, rhamnose, salicin, trehalose, citrate, urease, liquid indole, methyl-red, gelatin.

*Antimicrobial susceptibility* of the isolate was investigated by means of disc-diffusion test on the Müller-Hinton agar ("Torlak", Beograd, SCG) and using the following antibiotics: penicillin (10 µg), streptomycin (30 µg), neomycin (10 µg), trimetoprim-sulphamethoxazole (1.25 µg + 23.75 µg), ceftriaxone (30 µg), gentamicin (30 µg), amoxicillin (25 µg), ampicillin (10 µg), kanamycin (30 µg), linco-spectin (15 µg + 200 µg), tetracycline (30 µg) and enrofloxacin (5 µg).

#### RESULTS AND DISCUSSION

With respect to growth requirements, *L. monocytogenes* is quite simple and easily cultivated on standard laboratory media. The main problem arising on isolation of this bacterial species is mostly the very small number of organisms present in the samples. Hence selection of the tissue is of outmost importance, depending on the clinical form of the disease, e.g. spinal liquid, pons and medulla in encephalitis or placenta (cotyledons), fetal abomasal content and/or uterine discharge in abortions. Isolation of the agent is the most accurate diagnostic method; however, a high rate of isolation failures is typical for *L. monocytogenes*. Loeb (2004) performed retrospective analysis of 42 brain tissue samples of ruminants with signs of meningoencephalitis confirming the presence of Gram-positive bacteria in 47,6% of the cases and 80,9% of the cases were immunohistochemically positive for the listerial antigen. Bacteriological cultures were positive in 28,5% of the cases. Our investigation strongly suggests that bacteriological culture techniques are insufficient compared with immunohistochemistry for the confirmation of *Listeria monocytogenes* infection.

In our investigation we isolated *L. monocytogenes* from 18 of the total of 46 investigated tissue samples originating from animals with clinical diagnosis of listeriosis. Isolates (n=12) were cultured from samples of brain tissue and medulla oblongata of sheep which died with symptoms of CNS disease, and 6 from fetal tissue samples. *L. monocytogenes* was isolated from only two samples of brain tissue by the method of direct plating on blood agar. Isolates (n=16) were obtained after 7-14 days of enrichment in the Listeria enrichment broth base (Biolife) and incubation at 4°C and 30°C. Samples were simultaneously incubated at two temperatures (4°C and 30°C). Psychrophilic nature of *L. monocytogenes* allows its growth at refrigerator temperature. Incubation at a higher temperature shortened the isolation period for 2 to 5 days on average; however, it required purification, picking and subcultivation of the colonies (Walker *et al.*, 1990). Incubation at refrigerator temperature enabled isolation of *L. monocytogenes* in an almost pure culture, which is of outmost importance particularly if isolation is performed from highly contaminated samples.

On blood agar with 5% sheep blood *L. monocytogenes* formed small, round, translucent colonies surrounded by a zone of complete haemolysis, similar to β-haemolytic streptococci.

The ability of *Listeria* species to produce haemolysis is closely correlated with their pathogenicity, except for haemolytic non-pathogenic species *L. seeligeri*. Harvey and Faber (1941) first demonstrated the ability of *L.*

*monocytogenes* to produce soluble haemolysin, and Jenkins and Watson (1971) first time reported on the similarity of *Listeria* haemolysin and streptolysin O (SLO) of *Streptococcus pyogenes* with respect to their functionality and antigenic properties (Vazquez-Boland *et al.*, 2001). Haemolysin of *L. monocytogenes* is a cytolysin similar to SLO. It belongs to the family of cholesterol-dependent pore forming toxins (CDTX) that includes 23 known members of different genera of Gram-positive bacteria. The toxins are closely similar with respect to their composition, exhibiting strong antigenic cross-reactivity. This toxin was named listeriolysin (LLO). Subsequently, ivanolysin (haemolysin of *L. ivanovii*) was purified and it was confirmed that haemolytic, non-pathogenic species *L. seeligeri* produce an LLO- related CDTX, albeit in small amounts.

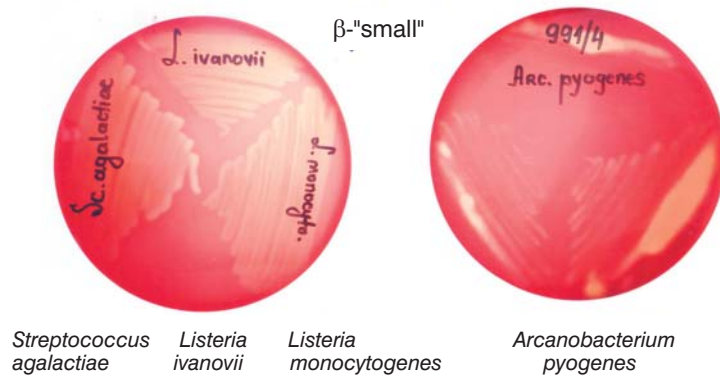


Figure 1. β-"small". Characteristic appearance of the complete lysis of sheep erythrocytes on blood agar – similarity of haemolytic colonies of *L. monocytogenes*, *L. ivanovii* and *Sc. agalactiae* (β). Right – haemolytic activity of *Arcanobacterium pyogenes*

On the ALOA agar *L. monocytogenes* formed small, bluish-green colonies surrounded by the opaque zone - the appearance clearly differentiating this species from other bacteria present in the sample.

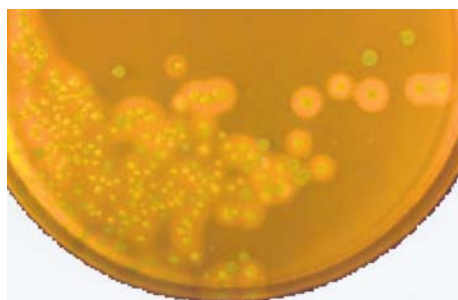


Figure 2. On the ALOA agar *L. monocytogenes* formed small, bluish-green colonies producing an opaque halo around the colonies

The selectivity of this solid medium is due to lithium chloride and to the addition of antimicrobial mixture containing ceftazidime, polymyxin B, nalidixic acid and cycloheximide. Differential activity of the medium is due to the presence of chromogenic compound for the detection of  $\beta$ -glucosidase, an enzyme common to all *Listeria* species. The specific differential activity is obtained by means of a substrate for a phosphatidylinositol-specific phospholipase C enzyme that is present only in *L. monocytogenes*

Further to haemolytic activity, the lecithinase activity is an easily recognizable phenotypic marker closely associated with the pathogenicity within the genus *Listeria*. Phospholipase production was first described by Fuzi and Pillis (1962), who observed opacity reactions in egg yolk agar that correlated in intensity with the hemolytic activity of the strains tested (Vazquez-Boland *et al.*, 2001). Pathogenic *Listeria* spp. produce three different enzymes with phospholipase C (PLC) activity that are involved in virulence. Two, PlcA and PlcB are present in *L. monocytogenes* and *L. ivanovii*, the third SmcL, is specific for *L. ivanovii*. SmcL is responsible for different haemolytic activities of *L. ivanovii*. This enzyme is functionally closely similar to the  $\beta$ -toxin of *S. aureus*. The sphingomyelinase of *L. ivanovii* exhibits lytic activity towards sheep erythrocytes, sphingomyelin making 51% of the total membrane lipids of their membrane.

Exposing such damaged erythrocytes to the soluble product released by *Rhodococcus equi* (a cholesterol-oxidase) results in more pronounced synergistic haemolytic effect, appearing as "closed umbrella". This appearance is often described as shovel or spade; however, such description is more adequate for the synergistic haemolysis of *S. aureus* and *Rh. equi* (Fig 3). This CAMP-like reaction is applied in routine practice for identification of *L. ivanovii*. Our *L. monocytogenes* isolates produced characteristic CAMP phenomenon with *Staphylococcus aureus*.



Figure 3. Synergistic (CAMP like) hemolysis of *L. ivanovii* and  $\beta$ -toxin (sphingomyelinase)-producing *S. aureus* with *R. equi* (vertical streak) Synergistic haemolyses reflects the functional relation of the sphingomyelinase C produced by these two bacteria. CAMP-like effect results from the total lysis of the halo of sphingomyelinase-damaged erythrocytes exposed to a cholesterol-oxidase from *R. equi*

*L. monocytogenes* isolates exhibited pronounced catalase positivity, oxidase negativity and ability to hydrolyze aesculin. Fermentation of glucose, maltose, rhamnose, salicin and trehalose was observed after 24 hours. Fermentation of lactose and saccharose was not evident until day 10. In this period fermenting activity toward other investigated parameters was not observed. Microscopy examination revealed uniformly distributed Gram-positive rods.

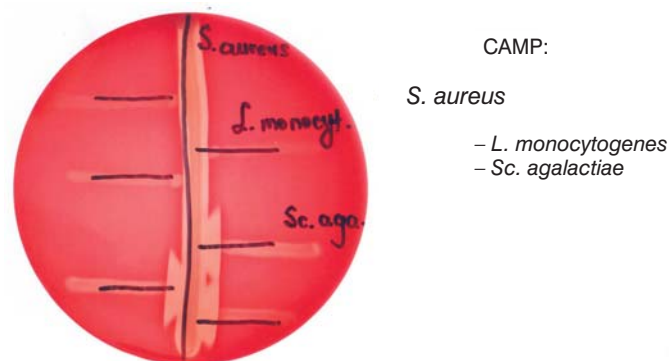


Figure 4. Positive CAMP test *L. monocytogenes* and *Staphylococcus aureus* (vertical). Listeriolysin (LLO) enhanced the effect of *S. aureus*  $\beta$ -toxin

Incubation at 25°C in gelatin resulted in characteristic growth zone (characteristic tumbling or "umbrella" motility). *Listeriae* are non-motile at 37°C, but the motility is evident on cultivation at 20-30°C. The motility of *L. monocytogenes* is a thermo-regulated phenomenon (Peel *et al.*, 1988). At temperatures below 30°C the organism is characterized by 4-6 peritrichous flagella (Schirm *et al.*, 2004). However, the increase of the temperature over 30°C results in reduced motility, and transcription of *flaA* appears to be completely stopped at 37°C, though minimal quantities of flagellin is immunologically detectable (Schirm *et al.*, 2004). Investigation of Peel *et al.* (1988) suggested that motility is down-regulated through the activity of PrfA, the transcription activator of the virulence gene.

Isolates exhibited susceptibility to penicillin, ampicillin, amoxicillin, tetracycline, gentamicin, trimetoprim-sulphametoxazole, kanamycin,, and lincospectin; intermediary susceptibility to neomycin, enrofloxacin and streptomycin, and resistance to ceftriaxone. In general, isolates of *L. monocytogenes*, as well as strains of other *Listeria* spp. are susceptible



Figure 5. Characteristic tumbling or "umbrella" motility *L. monocytogenes*

to a wide range of antibiotics except cephalosporins. The treatment of choice for listeriosis remains the administration of ampicillin or penicillin, combined with an aminoglycoside, usually gentamicin (Boisivon *et al.*, 1990). A clinical isolate of *L. monocytogenes* resistant to multiple antibiotics including gentamicin was reported (Tsakaris *et al.*, 1997). Resistance to other antibiotics such as streptomycin, erythromycin, kanamycin, sulfamethoxazole, or rifampin has also been observed (Charpentier and Courvalin, 1999).

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## IZOLACIJA *L. MONOCYTOGENES* KOD NERVNE FORME LISTERIOZE I ABORTUSA PREŽIVARA

VIDIĆ BRANKA, MILANOV DUBRAVKA I BUGARSKI D

### SADRŽAJ

*Listeria monocytogenes* je hranom prenosivi patogen, uzročnik teških infekcija ljudi i životinja koje se mogu manifestovati u formi abortusa, septikemije, meningitisa i meningoencefalitisa. Zbog varijabilnih kliničkih manifestacija, izolacija uzročnika je najsigurnija potvrda listerioze. Direktna izolacija *L. monocytogenes* relativno je laka kada je broj ovih mikroorganizama visok kao kod septikemične forme bolesti, ali je izolovanje teško ako je uzročnik prisutan u malom broju kao u slučaju encefalitisa ili ako je uzorak jako kontaminiran. U ovom radu je prikazana izolacija *L. monocytogenes* upotrebom selektivnih podloga za obogaćenje i izolaciju, u kombinaciji sa tehnikom hladnog obogaćenja. *L. monocytogenes* je izolovana u 18 od 46 pregledanih uzoraka tkiva preživara kod kojih je listerioza bila klinički suspektna. Takođe je prikazan i osnovni diferencijalno-dijagnostički postupak u odnosu na imitirajuće bakterijske vrste.