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Clinico-Pathological, Bacteriological and PCR Findings of Ovine Listeriosis: An Emerging Disease in Southern Iran

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Abstract: In this study, listeriosis was reported as an emerging disease in southern Iran. A combined histopathological, bacteriological and PCR method was conducted for the diagnosis of *Listeria monocytogenes*. *L. monocytogenes* was recovered from the brain of affected sheep with clinical signs of listerial encephalitis. The source of contamination was spoiled corn silage with pH 7.2. Based on the clinical findings of brainstem dysfunction and cranial nerve involvements, histopathology, bacteriology and PCR findings, the final diagnosis of listeric encephalitis due to *L. monocytogenes* infection was made. Because of the long period of drought in the region, the usage of corn silage was favoured and it seems that the possibility of the occurrence of clinical disease has been increased. As the risk of disease in human may increase with higher infectious doses of the organism, surveillance on the occurrence of the listeriosis in humans should be considered in public health programs.

Key words: Listeriosis · Encephalitis · Sheep · Histopathology · PCR · Iran

INTRODUCTION

Listeriosis usually results from infection by *Listeria monocytogenes*, a Gram positive rod in the family Listeriaceae. This intracellular organism is a small facultatively anaerobic, nonsporulating rod, which produces encephalitis, septicemia and abortion in both humans and livestock. Livestock are susceptible to listeric infection, with a large proportion of healthy asymptomatic animals shedding *L. monocytogenes* in their feces. There are 13 serovars of *L. monocytogenes*. Although all are considered to be potentially virulent, serovars 4b, 1/2b and 1/2a cause most animal and human disease. *L. ivanovii* (formerly known as *L. bulgarica* or serovar 5 of *L. monocytogenes*) is occasionally associated with abortions in sheep and cows, or septicemia in sheep [1 - 3]. The data available to date suggest that silage-fed ruminants and the associated farm environment can maintain high *L. monocytogenes* densities, most likely due to a combination of multiple factors, including *L. monocytogenes* growth in poorly fermented silage as well as fecal shedding by animals. *L. monocytogenes* that are fecally shed are dispersed into the environment, e.g., onto plant material that may be used for subsequent silage production, thus effectively maintaining an infectious cycle. Interestingly, clinical disease in ruminant populations that are fed silage year round appears to be uncommon and is generally limited to a single or few animals in a herd if disease occurs. In these ruminant populations, disease cases often appear to be linked to either consumption of silage contaminated with extremely high levels of *L. monocytogenes* or immunosuppression or both [4, 5]. Listeriosis is a primary cause of disease in cattle and sheep [6]. Listeria can also cause mastitis [6, 7] and metritis [7] as well as iritis and keratoconjunctivitis [8]. Encephalitis and reproductive disease forms are the most common clinical presentations [9]. In addition to ruminant species, *L. monocytogenes* also can be isolated from a number of nonruminant species and nonruminant agricultural environments. For example, *L. monocytogenes* has been isolated from the feces of wild birds [10], horses [2, 11], swine [12, 13], poultry [2] and other domestic animals [2] as well as from eviscerated farmed fish [14]. This paper described the first clinical listeric encephalitis as an emerging disease in southern Iran. Clinico-pathological, bacteriological and PCR findings were included.

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MATERIALS AND METHODS

In January 2009, a group of five male and female sheep aged between 1.5 to 3.5 years old were referred from two herds of 345 sheep in southern Iran, Fars Province (Doroudzan region) to Shiraz Veterinary Teaching Hospital. The animals exhibited a variety of clinical signs including anorexia, recumbency, facial paralysis, tongue protrusion, lip paralysis and saliva drooling (Figure 1). The herds were vaccinated against contagious agalactia, sheep pox, *Clostridium perfringens* type D and anthrax. The sheep were fed alfalfa hay, corn silage and different proportions of concentrates. All the affected sheep underwent a comprehensive clinical and neurological examination by the methods described by Lorenz and Kornegay [15]. All the affected sheep were euthanized. At necropsy, no significant gross lesions were seen in the thoracic and abdominal organs. Five days later, eight other cases were referred from the same herds with the similar clinical signs. Corn silage that was shared between herds was spoiled and had the pH of 7.2 and it was assumed that the outbreak was caused by feeding high pH silage. Based on the tentative diagnosis of listeriosis, treatment was initiated with penicillin at a dosage of 44000 IU/kg BW given intramuscularly daily for 10 days in latter cases and feeding of spoiled silage was stopped. The treatment was successful. Tissues (brain, ileum, duodenum, liver and kidney) and serum were obtained from the euthanized sheep. Histopathology, bacteriology and PCR were performed. Three tissue samples of about 1 cm^3 in thickness from each of the cerebrum, cerebellum, medulla oblongata, proximal, middle and distal parts of spinal cord were fixed in 10% neutral buffered formalin. These samples were then dehydrated in graded ethanol, embedded in paraffin. Sections of 5 µm in thickness were stained with hematoxylin and eosin and examined by an ordinary light microscope. For bacteriological examination a brain sample of 2 g was added to 10 mL of Brain heart infusion broth (Merck 1.10493). Samples were homogenized with a stomacher (Seward stomacher 400, Seward, UK) for 30–60 s and then incubated at 30°C for 24 h. This served as an enrichment phase. This enriched material were subcultured on to blood agar and PALCAM- Listeria selective agar (Merck) with PALCAM-listeria selective supplement and incubated at 35°C for 24 h. Presumptive *L. monocytogenes* colonies were

Fig. 1: Facial paralysis, lip paralysis and saliva drooling in listeric encephalitis affected sheep

streaked on nutrient agar and incubated at 37°C for 18–24 h. The colonies on nutrient agar identified using morphological, cultural and biochemical criteria. In particular, Gram stain, catalase test, mobility, â-hemolysis and production of acids from rhamnose and xylose were used, as described in Bergey's manual [16] to identify the species belonging to the genus. For the PCR assays the extraction of DNA was performed. The extraction procedure was based on a protocol previously described for the detection of *L. monocytogenes* in food products [16]. A few colonies were resuspended in 50 µL of 1×PCR buffer in a 2mL microcentrifuge tube with an interlocking cap. A solution of 2 % Triton X (50 µL) was then added to this cell suspension and thoroughly mixed. This mixture was heated at 100°C for 10 min and then allowed to cool to room temperature. For PCR amplification, 5 µL of this crude cell lysate were used. PCR assays were performed in 25 µL reaction volumes. The primer pair consisting 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphates, 1.25 IU of *Taq* polymerase (Fermentas, Germany) and 0.2 mM (each) primers targeting the *hlyA* gene encoding the hemolysin A in *L. monocytogenes*. Primers were named primer A [5`-CCT AAG ACG CCA ATC GAA-3`] ?and primer B [5`-TAG TTC TAC ATC ACC TGA GAC AGA-3`]? was used for the amplification of a 840 bp region of the *hlyA* gene [17; 18]. PCR was performed in the Perkin Elmer GeneAmp PCR system 2400 thermal cycler. Amplification conditions were optimized to the thermal cycler and were as follows: an initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 50 s, annealing at 64°C for 50 s and extension at 72°C for 60 s, then a final extension at 72°C for 7 min. The amplified DNA was analyzed by gel electrophoresis on a 1.2 % agarose gel stained with ethidium bromide (3mL/100mL). A 100 bp ladder (Fermentas) was used as a reference marker. Tris-borate EDTA (0.5×) was used as the running buffer and the gel was viewed using UV transillumination at a wavelength of 254 nm.

RESULTS

Histopathology: The main microscopic changes comprised of asymmetrical meningoencephalitis with severe hyperemia and parenchymal degeneration centered in the pons and medulla oblongata, particularly in the nucleus and proximal part of the spinal tract. Disseminated intravascular coagulation was evident in some of the capillaries and venules. Inflammation in the degenerated neuroparenchyma was marked by prominent perivascular cuffs of inflammatory cells that include lymphocytes, plasma cells, macrophages and fewer neutrophils (Figs. 2 and 3). Similar cell populations were found in the leptomeninges adjacent to the areas of parenchymal injury (Fig. 4) and in lower number in cerebellar parenchyma and other parts of central nervous system except middle and caudal parts of the spinal cord.

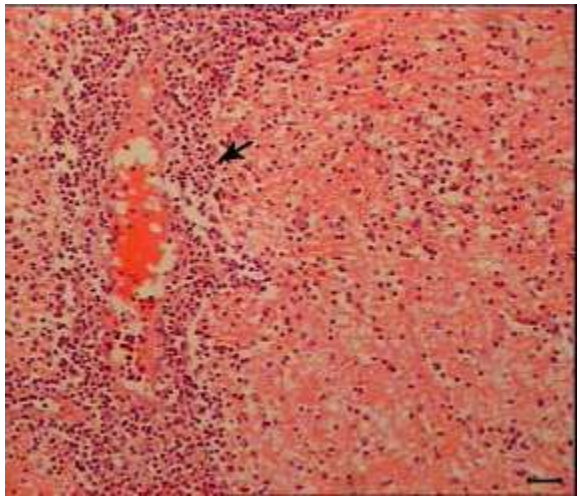

Fig. 2: Tissue section from the cerebrum of an infected sheep with listeriosis. Perivascular cuffing with lymphocytic infiltration is evident in this section. Lymphocytes are also infiltrated in the brain parenchyma (H and E, scale bar=85 µm)

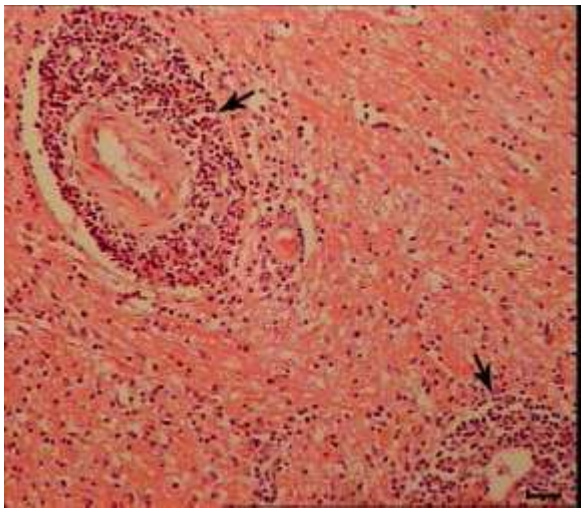

Fig. 3: Tissue section from the cerebrum of an infected sheep with listeriosis. Perivascular cuffing with lymphocytic infiltration is evident around blood vessels of this section (arrows). Lymphocytes are also diffusely infiltrated in the brain parenchyma (H and E, scale bar=85 µm)

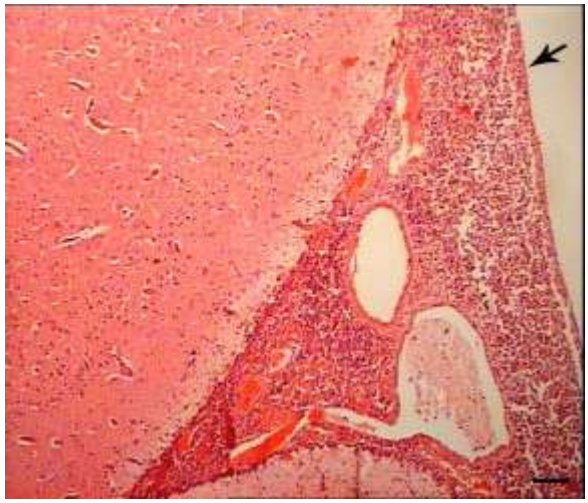

Fig. 4: Tissue section from the cerebellum of an infected sheep with listeriosis. Meningoencephalitis with neutrophil, lymphocyte, plasma cell and macrophage infiltration is evident in the meninges and peripheral parenchyma (H and E, scale bar=160 µm)

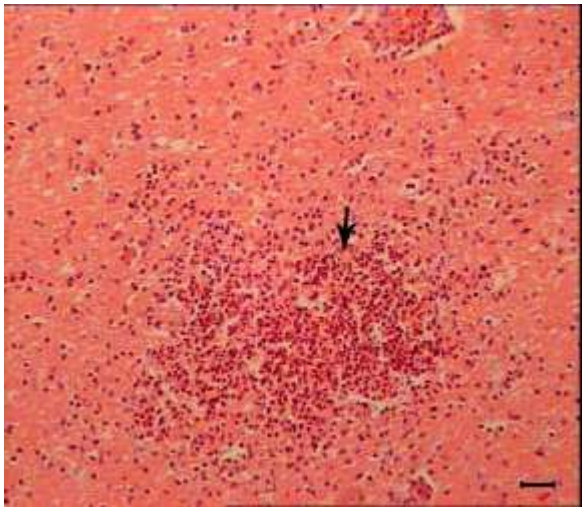

Fig. 5: Tissue section from the cerebrum of an infected sheep with listeriosis. A micro abscess is present in the brain parenchyma (arrow). Neutrophils and lymphocytes with tissue necrosis are evident in the parenchyma (H and E, scale bar=85 µm)

Typically the inflammation consisted of a mixture of lymphocytes, plasma cells and macrophages as nonsupporative cells and neutrophils as suppurative ones. Microabscesses were occasionally observed in the periphery of the cerebral and cerebellar parenchyma (Fig. 5). Bacteriology and PCR. The bacterial culture from the brain, confirmed to be *L. monocytogenes* by means of

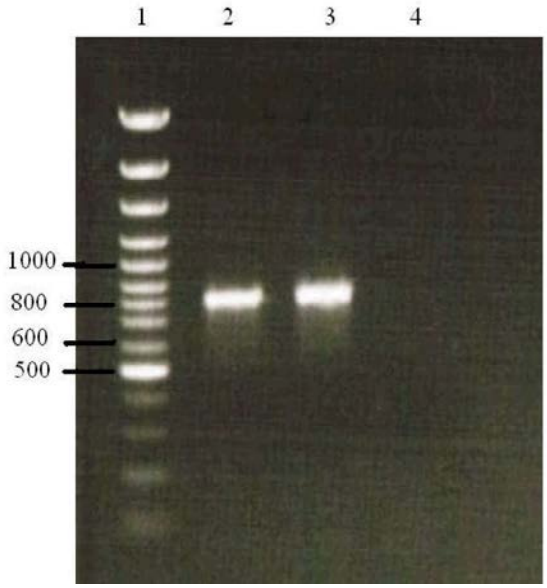

Fig. 6: Agarose gel electrophoresis of the PCR amplification products of hlyA gene of *Listeria monocytogenes* isolated from brain. Lane 1, 100bp DNA ladder, Lane 2 isolated sample, Lane 3 Positive sample of *L. monocytogenes* and Lane 4 negative control

biochemical tests. The positive culture of *L. monocytogenes* in biochemical tests was confirmed by PCR (Fig. 6). Based on clinical findings of brainstem dysfunction and cranial nerve involvements, histopathology, bacteriology and PCR findings, the final diagnosis of listeric encephalitis due to *L. monocytogenes* was made.

DISCUSSION

The diagnosis of listeric encephalitis in affected sheep was based on the clinical findings of brainstem dysfunction and cranial nerve involvements, histopathology, bacteriology and PCR [17 - 19]. Typically there are no distinctive gross changes associated with listerial encephalitis [19]. Histological examination of central nervous system tissue is necessary to demonstrate the microabscesses that are characteristic of the disease [19]. PCR is deemed to be more reliable than conventional identification since it is based on stable genotypic characteristics rather than relying on biochemical or physiological traits, which can be genetically unstable [20]. With bacterial adaptation to different environments causing similarities in phenotype, as well as resistance to ingredients in enrichment and

selective media, the transition from conventional methods of detection to genetic methods should be carried out [17, 18]. The PCR method proved to be reliable, cost effective and time saving [20]. The combined use of histopathology, bacteriology and PCR detection of Listeria seems to allow an effective detection of *L. monocytogenes* infection in sheep in our study. To the best of authors' knowledge this is the first clinical report of ovine listeric encephalitis in Iran. There are few reports of the disease in other countries of the region in human and animals [21-23]. Listeriosis caused by *L. monocytogenes* is an infectious disease affecting a wide range of mammals, including ruminants, monogastric animals and humans. In ruminants, among which sheep are the ones more commonly affected [24]. While, the majority of infections are subclinical, listeriosis in animals can occur either sporadically or as epidemics [25]. Outbreaks do occur when multiple animals are exposed to a single contaminated source, such as silage [26]. Due to long period of drought (2007 to 2009) in the region, the usage of silage was favoured and it seems that the possibility of the occurrence of clinical disease has been increased due to the probable feeding of spoiled silages. This is why that there has been no report from Iran till now. Another reason may be due to fastidious nature of the organism and limitation on application of specific diagnostic methods. Reports of listeriosis in domestic animals have increased worldwide, including in England, New Zealand, Germany and Eastern Europe, which may reflect fluctuations in the distribution of susceptible hosts, changes in silage production and /or improved detection methods [27]. 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 [28, 29]. Silage feeding is also considered to have some intrinsic immunosuppressive effect in sheep [28, 29]. Although the organism has been isolated from the milk in Iran [30], the isolation of the organism from clinically affected animals have not been reported. *L. monocytogenes* is part of the normal flora of the distal part of the intestinal tract of numerous animal species and has been isolated from mammalian species, birds, fish and also ticks or flies [28]. But *L. monocytogenes* seems to have a lower pathogenic potential than other food-borne pathogens [28]. The lack of clinical cases in neighbor herds may be due to different types of ration other than silage. The occurrence of the disease in the two herds in winter (January) may be associated with silage feeding and/or because Listeria can grow at temperatures where growth of other pathogens is inhibited due to excessive cold [26]. The excretion of *L. monocytogenes* by sheep has been linked to diet, especially consumption of poorly prepared silage [1, 31, 32]. In Great Britain, a significant association between silage feeding and an increased relative risk for development of ovine listeric encephalitis was determined [33, 34]. Silage fermentation involves the action of lactic acid bacteria on crops with high moisture content under anaerobic conditions. However, as silage is exposed to air during storage or at feeding time, aerobic spoilage occurs promptly due to lactic acid degradation by the mainly lactic acid-utilizing yeasts [35]. This consequently leads to an increase in pH of the silage [36]. *L. monocytogenes* is commonly present in silage, but it can multiply only in silage poorly fermented above a pH 5.0–5.5. Outbreaks of listeriosis can also occur without feeding the silage, but with poor quality pastures or rotting vegetation [37, 38]. In view of the fact that the disease has potential zoonotic importance, the level of infection in animals may have significant effect on the occurrence of the disease in humans. As the clinical disease was observed during the drought period in the region, it seems that the carrier animals and shedding of the *Listeria* organism may also increase. As the risk of disease in human may increase with higher infectious doses of the organism, surveillance on the occurrence of the listeriosis in humans should be considered in public health programs [39]. It was concluded that the disease is present in the region and the risk of the disease should be kept in mind and be aware of clinical signs and surveillance on the disease should be considered.

REFERENCES

1. Gray, M.L. and A.H. Killinger, 1966. Listeria monocytogenes and listeric infections. Bacteriol. Rev., 30: 309-382.
2. Weber, A., J. Potel, R. Schaefer-Schmidt, A. Prell and C. Datzmann, 1995. Investigations on the occurrence of L. monocytogenes in fecal samples of domestic and companion animals. Zentralbl. Hyg. Umweltmed., 198: 117-123.
3. Lorber, B., 2007. Listeriosis. In Listeria monocytogenes: Pathogenesis and Host Response, Eds., Goldfine, H., H. Shen, New York, Springer, pp: 13-32.
4. Wesley, I.V. 1999. Listeriosis in Animals. In: Listeria, listeriosis and food safety. Ryser, E.T., E.H. Marth, Eds., New York, Marcel Dekker. PP: 39–74.

5. Roberts, A.J. and M. Wiedmann, 2003. Pathogen, host and environmental factors contributing to the pathogenesis of listeriosis. Cell. Mol. Life. Sci., 60: 904-918.
6. Cooper, J. and R.D. Walker, 1998. Listeriosis. Vet. Clin. North. Am. Food. Anim. Pract., 14: 113-125.
7. Sanchez, S., M. Studer, P. Currin, P. Barlett and D. Bounous, 2001. Listeria keratitis in a horse. Vet. Ophthamol., 4: 217-219.
8. Erdogan, H.M., B. Cetinkaya, L.E. Green, P.J. Cripps and K.L. Morgan, 2001. Prevalence, incidence, signs and treatment of clinical listeriosis in dairy cattle in England. Vet. Rec., 149: 289-293.
9. Low, J.C. and W. Donachie, 1997. A review of Listeria monocytogenes and listeriosis. Vet. J., 153: 9-29.
10. Fenlon, D.R., 1985. Wild birds and silage as reservoirs of Listeria in the agricultural environment. J. Appl. Bacteriol., 59: 537-543.
11. Gudmundsdottir, K.B., V. Svansson, B. Aalbaek, E. Gunnarsson and S. Sigurdarson, 2004. Listeria monocytogenes in horses in Iceland. Vet. Rec., 155: 456-9.
12. Hayashidani, H., N. Kanzaki, Y. Kaneko, A.T. Okatani, T. Taniguchi, K. Kaneko and M. Ogawa, 2002. Occurrence of yersiniosis and listeriosis in wild boars in Japan. J. Wildl. Dis., 38: 202-5.
13. Yokoyama, E., T. Saitoh, S. Maruyama and Y. Katsube, 2005. The marked increase of Listeria monocytogenes isolation from contents of swine cecum. Comp. Immunol. Microbiol. Infect. Dis., 28: 259-268.
14. Miettinen, H. and G.Wirtanen, 2005. Prevalence and location of Listeria monocytogenes in farmed rainbow trout. Int. J. Food. Microbiol., 104: 135-143.
15. Lorenz, M.D. and J.N. Kornegay, 2004. Neurologic history and examination. In Handbook of Veterinary Neurology, Lorenz, M.D. and J.N. Kornegay, Eds., Philadelphia, W. B. Saunders, pp: 3-44.
16. Blais, B.W., L.M. Phillippe, M. Burzynski and J. Bielecki, 1995. Applicability of the PCR technique in the food testing laboratory: Identification of Listeria monocytogenes. Biotechnol. Tech., 9: 629-632.
17. Border, P., J. Howard, G. Plastow and K. Siggens, 1990. Detection of Listeria species and Listeria monocytogenes using polymerase chain reaction. Lett. Appl. Microbiol., 11: 158-162.
18. Bsat, N. and C. Batt, 1993. A combined modified reverse dot-blot and nested PCR assay for the specific non-radioactive detection of Listeria monocytogenes. Molec. Cellul. Probes., 7: 199-207.
19. Johnson, G.C., W.H. Fales, C.W. Maddox and J.A. Ramos-Vara, 1995. Evaluation of laboratory tests for confirming the diagnosis of encephalitic listeriosis in ruminants. J. Vet. Diagn. Invest., 7: 223.
20. Lawrence, L.M. and A. Gilmour, 1994. Incidence of Listeria spp. and Listeria monocytogenes in a poultry processing plant environment and in poultry products and their rapid confirmation by multiplex PCR. Appl. Environ. Microbiol., 60: 4600-4604.
21. Nasim, R.B. and R. Vahidy, 1993. Human listerial meningitis: reported from karachi, Pakistan. J. Islamic. Acad. Sci., 6: 253-258.
22. Sahin, M. and E. Beytut, 2006. Abortions in sheep due to Listeria ivanovii in the Kars region. Turk. J. Vet. Anim. Sci., 30: 503-506.
23. Yousif, Y.A., B.P. Joshi and H.A. Ali, 1984. Ovine and caprine listeric encephalitis in Iraq. Trop. Anim. Health. Prod., 16: 27-28.
24. Brugère-Picouxa, J., 2008. Ovine listeriosis. Small Rumin. Res., 76: 12-20.
25. Kimberling, C.V., 1988. Diseases of the central nervous system. In Diseases of sheep, R. Jensen and B.L. Swift, Eds., Philadelphia, Lea and Febiger, pp: 195-199.
26. George, L.W., 2009. Listeriosis. In Large Animal Internal Medicine Ed., Smith, B.P., (4th Ed). St Louis, Mosby, pp: 1045-1048.
27. Anonymous, A., 1988. FDA regional workshops to discuss microbial concerns in seafood. Food. Chem. News., 30: 27.
28. Audurier, A., P. Pardon, J. Marly and F. Lantier, 1980. Experimental infection of mice with Listeria monocytogenes and L. innocua. Ann. Microbiol., 131: 47-57.
29. Wagner, M., D. Melzner, Z. Bago, P. Winter, M. Egerbacher, F. Schilcher, A. Zangana and D. Schoder, 2005. Outbreak of clinical listeriosis in sheep: evaluation from possible contamination routes from feed to raw produce and humans. J. Vet. Med. B., 52: 278-283.
30. Jalali, M. and D. Abedi, 2008. Prevalence of Listeria species in food products in Isfahan, Iran. Int. J. Food. Microbiol., 122: 336-340.
31. Blenden, D.C., E.H. Kampelmacher and M.J. Torres-Angel, 1987. Listeriosis. J. Am. Vet. Med. Assoc., 191: 1546-1551.

32. Vazquez-Boland, J.A., L. Dominguez, M. Blanco, J. Rocourt, J.F. Fernandez-Garyzabal, C.B. Gutierrez, R.I. Tascon and E.F. Rodriguez-Ferri, 1992. Epidemiologic investigation of a silage-associated epizootic of ovine listeric encephalitis, using a new Listeria selective enumeration medium and phage typing. Am. J. Vet. Res., 3: 368-371.
33. Beauregard, M. and K.L. Malkin, 1971. Isolation of Listeria monocytogenes from brain specimens of domestic animals in Ontario. Can. Vet. J., 12: 221-223.
34. Schweizer, G., F. Ehrensperger, P. Torgerson and U. Braun, 2006. Clinical findings and treatment of 94 cattle presumptively diagnosed with listeriosis. Vet. Rec., 158: 588-592.
35. Sasaki, H., 1972. Microbiological studies on grass silage fermentation. Mem. Fac. Agric. Hokkaido University, 8: 189-251.
36. Woolford, M.K., 1990. A review: the detrimental effects of air on silage. J. Appl. Bacteriol., 68: 101-116.
37. Kumar, H., B.B. Singh, M.S. Bal, K. Kaur, R. Singh, P.K. Sidhu and K.S. Sandhu, 2007. Pathological and epidemiological investigations into Listeria encephalitis in sheep. Small. Rumin. Res., 71: 293-297.
38. Radostits, O.M., C.C. Gay, K.W. Hinchcliff and P.D. Constable, 2007. Veterinary Medicine. A Textbook of the Disease of Cattle, Horses, Sheep, Pigs and Goats. 10th Ed, London, Saunders, pp: 805-810.
39. Regan, E.J., G.A.J. Harrison, S. Butler, J. McLauchlin, M. Thomas and S. Mitchell, 2005. Primary cutaneous listeriosis in a veterinarian. Vet. Rec., 157: 207.