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RESEARCH ARTICLE

Encephalitic Listeriosis in Small Ruminants in Oman: Pathophysiology, Antimicrobial Sensitivity and Molecular Characterization

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A B S T R A C T

Listeriosis is an infectious and fatal disease of domestic mammals and humans caused by *Listeria monocytogenes* (*L. monocytogenes*). This study discusses the prevalence, pathology, microbiology, and molecular characterization of reported encephalitic listeriosis cases in caprine and ovine species in the north of Oman between 2017 and 2019. Thirty suspected small ruminants (goats=20 and sheep=10) were reported from eight outbreaks in northern Oman during the 3 years. The overall morbidity, mortality, and case fatality in goats were 5.8, 5.5 and 95%, respectively, while in sheep, were 8.4, 4.2 and 50%, respectively. Animals under 12 months of age had a case fatality rate of 100% compared to 50% in animals older than one year. The clinical signs in all reported cases were fever, anorexia, deviated neck, and circling. Necropsied animals showed no significant gross lesions. Microscopically, microabscesses, hemorrhages, and perivascular cuffs were observed in the hindbrain along with positive immunostaining of intracellular *L. monocytogens* antigens in the mononuclear and polymorph nuclear cells. Two *L. monocytogenes* isolates (A4 and E7) from two goats had multiple antimicrobial-resistant indices scores of 0.5 and 0.3 respectively which is above the Krumperman permissible threshold (>0.2), indicating a high-risk source of contamination by the resistant antibiotics in the study area. Encephalitic listeriosis was further confirmed through the 16sRNA molecular characterization and phylogenetic analysis of the two listeria isolates. Here we report two multidrug-resistant *L. monocytogens* isolates from goats in Oman, which pose a serious public health threat to both humans and animals.

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INTRODUCTION

Listeriosis is an infectious and fatal disease of domestic mammals, especially ruminants, poultry, and humans. It is caused by the bacterium *Listeria monocytogenes* (*L. monocytogenes*), an aerobic and facultative anaerobic, intracellular, and Gram-positive rods (Dhama *et al.*, 2013; Craig *et al.*, 2019). It's a worldwide disease that can occur in a sporadic or epidemic form (Swaminathan and Gerner-Smidt, 2007; Desai *et al.*, 2019). Listeriosis has a public health significance as a food-borne zoonotic disease and it can survive refrigeration temperatures, acidic pH, and high salt concentrations (Dhama *et al.*, 2015; McLauchlin *et al.*, 2020). *L. monocytogenes* can cause severe disease in humans, immunocompromised and elderly patients, pregnant women, and newly born babies (Motarjemi and Adams, 2006). Listeriosis is a rare disease in human

beings but has a high case-fatality rate of 20–50% (Swaminathan and Gerner-Smidt, 2007). Clinically, the disease is commonly associated with gastroenteritis, septicemia, and encephalitis (Swaminathan and Gerner-Smidt, 2007; Schaefer *et al.*, 2022).

In ruminants, the disease is frequent in winter and spring and related to feeding of contaminated silage; hence the name "Silage Sickness" (McGavin *et al.*, 2001; Ramaswamy *et al.*, 2007). Listeria has three distinct clinical forms: encephalitis, septicemia, and abortion (Brugère-Picoux, 2008; Fentahun and Fresebehat, 2012). The encephalitic form is the commonest and is frequently fatal, especially in sheep, with a mortality rate of 5-10% (Brugère-Picoux, 2008; Scott, 2013). Although the prevalence of listeriosis in small ruminants was 28.5%, meningoencephalitis was reported at a lower rate of 4.7% (Loeb, 2004). The associated neuropathological changes are microabscesses formation in the hindbrain, which manifested clinically by unilateral ataxia, known as "circling disease" (Mansbridge *et al.*, 2018). Early diagnosis and appropriate antimicrobial therapy are crucial to survival and reduction of neurological sequelae of encephalitic cases (Reynaud *et al.*, 2007; Wei *et al.*, 2020).

Scarce data is available on listeriosis in Oman. However, Oman received listeria-contaminated cantaloupe melons from Australia in 2018 (Desai *et al.*, 2019). The current study discusses the isolation, pathology, molecular characterization, and antimicrobial sensitivity of reported encephalitic listeriosis in caprine and ovine in northern Oman between 2017 and 2019.

MATERIALS AND METHODS

Study animals: The current study was conducted over 3 years between January 2017 and December 2019. All veterinary practices in Oman were instructed to report any suspected encephalitic listeriosis in ruminants by the General Directorate of Animal Wealth at the Ministry of Agriculture, Fisheries, and Water Resources. A case was suspected to be encephalitic listeriosis when the animal shows fever, aimless movement with a deviated neck, and circling (Loeb, 2004; Fentahun and Fresebehat, 2012).

Clinical examinations and sampling collections: The local veterinarians clinically examined the suspected animals throughout the disease. Clinical signs were recorded. Serum samples and carcasses were immediately sent to the Central Laboratory for Animal Health (CLAH), Muscat for serological, pathological, and bacteriological examination.

Serology: All serum samples were screened for caprine arthritis encephalitis (CAE) virus antibodies by ID Screen® ELISA (ID.VET Innovative Diagnostics, Grabels, France) according to manufacturer instructions.

Pathology: Necropsy of all dead suspected cases was performed. The brain and spinal cord were thoroughly examined for apparent lesions that may cause nervous manifestations. The brains were examined with ultraviolet light illumination for polioencephalomalacia. Direct brain stem smears were Gram stained for bacterial detection (Markey *et al.*, 2013). Brain samples were collected and fixed in 10 % neutral buffered formalin then routinely processed and stained with hematoxylin and eosin (H&E) (Culling, 2013).

Consecutive paraffin sections were stained immunohistochemically using rabbit polyclonal Anti-*L. monocytogenes* primary antibody (ab35132; Abcam) at 1:100 dilution followed by horseradish peroxidase-linked anti-rabbit IgG secondary antibody and developed with 3,3′-diaminobenzidine peroxidase (DAB) substrate (MP-7800, ImmPRESS® Universal PLUS Polymer Kit, Vector Laboratories). The primary antibody was omitted from the negative control slides and a tissue section from a confirmed encephalitic listeriosis case was used as a positive control.

Bacteriological investigations: The samples from the brain stem (midbrain, pons, and medulla oblongata) were aseptically collected upon necropsy, immediately inoculated aerobically on Columbia sheep blood agar (ThermoFisher Scientific, UK) and incubated overnight at 37°C. Bacteria were stained with Gram stain and identified by VITEK® two system (bioMérieux, USA). The antibiogram analysis was performed using VITEK® 2 GP ID (bioMérieux cards), complementing the diffusion disk method. The antibiogram results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) criteria. Multiple Antimicrobial Resistance Indices (MARI) were calculated as the ratio of the number of antibiotics to which an organism is resistant to the total number of antibiotics to which the organism is exposed. MARI values greater than 0.2 indicate a high-risk source of contamination where antibiotics are usually used (Woh *et al.*, 2023).

DNA extraction and molecular identification: DNA extraction was performed using DNAzol® Direct reagent (# DN 131, MRC, USA) following the manufacturer's directions and a 1:10 dilution of the mixture using MilliQ water was used directly in the PCR master mix.

Two universal primers (27F, 1492R) were used to amplify the 16S rRNA gene fragment following (Frank *et al.*, 2008). The sequencing reaction product was isolated using sodium acetate and analyzed using a genetic analyzer in 96-Well Plates (3130 XL, Applied Biosystems, USA). The sequencing findings were compared to those in the National Center for Biotechnology Information's (NCBI) nucleotide database.

Phylogenetic analysis: A phylogenetic tree based on 16S rRNA gene sequences was built using additional isolates from GenBank. TREECON (Van de Peer and De Wachter, 1994) was used to measure evolutionary distances using the Tamura–Nei model. Molecular Evolutionary Genetics Analysis (MEGA version 6) software was used to perform parsimony and maximum likelihood (ML) studies (Tamura *et al.*, 2013). Bootstrap simulations of 1000 replicates were used to determine the durability of assumed tree topologies.

RESULTS

Study animals: Thirty suspected small ruminants (goats=20 and sheep=10) were reported over eight

Table 1: Reported outbreaks of encephalitic listeriosis in small ruminants in Oman (2017-2019).

Parameter	Outbreaks							
	1	2	3	4	5	6	7	8
Date	July 2017	August 2017	September 2018	August 2019	August 2019	October 2019	October 2019	November 2019
Species	Caprine	Ovine	Ovine	Caprine	Caprine	Caprine	Caprine	Caprine
Location	Bidbid/ADakhliya	Barka/AlBatinah	Barka/AlBatinah	A Seeb/Muscat	A Seeb/Muscat	Baushar/Muscat	A Seeb/Muscat	A Seeb/Muscat
No. of animals/farm	56	77	42	140	30	40	70	8
Diseased	3	6	4	4	6	2	2	3
Dead	3	4	1	4	6	1	2	3
Fatality%	100%	66.7%	%25	100%	100%	50%	100%	100%
Age	<6 months	1-3 Years	2-3 Years	≤1 Year	≤ 1 Year	1-2 years	≤ 1 Year	≤ 1 Year

Table 2: Summary of clinical signs of encephalitis listeriosis in small ruminants in Oman (2017-2019).

Clinical signs	Outbreaks							
	1	2	3	4	5	6	7	8
Temperature	39-40 °C	40-41°C	40-41°C	39-40°C	40-41°C	40°C	39-40°C	40-42°C
Anorexia	3/3	6/6	4/4	4/4	6/6	2/2	2/2	3/3
Deviated neck	3/3	6/6	4/4	4/4	6/6	2/2	2/2	3/3
Circling	3/3	6/6	4/4	4/4	6/6	2/2	2/2	3/3
Loss of corneal reflex	1/3	6/6	2/4	0/4	0/6	2/2	0/2	1/3
Dropping ear	3/3	6/6	2/4	4/4	2/6	2/2	0/2	1/3
Protruded tongue	0/3	6/6	1/4	4/4	2/6	2/2	1/2	0/3
Hanging jaw/ drooling saliva	0/3	6/6	1/4	4/4	2/6	2/2	1/2	0/3

Table 3: Summary of neuropathological results of encephalitis listeriosis in small ruminants in Oman (2017-2019).

Neuropathology	Outbreaks							
	1	2	3	4	5	6	7	8
Brain stem	3/3	4/4	1/1	4/4	6/6	1/1	2/2	3/3
Intracellular Gram-positive rods								
Microabscesses	3/3	4/4	1/1	4/4	6/6	1/1	2/2	3/3
Perivascular cuffing	3/3	4/4	1/1	4/4	6/6	1/1	2/2	3/3
Neuronal degeneration	3/3	4/4	1/1	4/4	6/6	1/1	2/2	3/3
Hemorrhages	1/3	2/4	0/1	3/4	2/6	1/1	1/2	2/3
Meningitis	0/3	1/3	0/1	2/4	1/6	0/1	0/2	1/3
Cerebellum								
Purkinje cells degeneration	2/3	3/3	1/1	3/4	4/6	1/1	1/2	1/3
Meningitis	0/3	0/3	1/1	1/4	2/6	0/1	0/2	0/3
Anterior spinal cord								
Microabscesses	1/3	0/3	0/1	1/4	1/6	/10	0/2	0/3
Meningitis	2/3	3/4	1/1	2/4	3/6	1/1	2/2	2/3

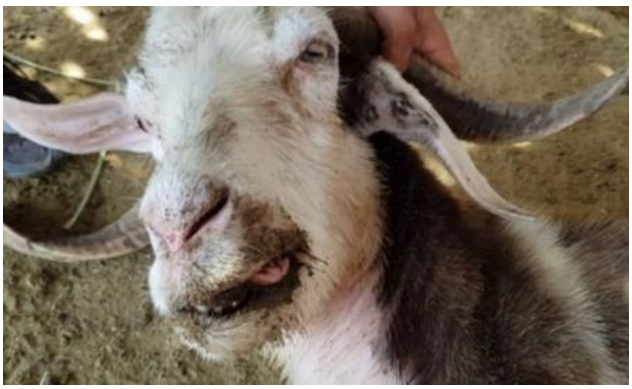

Fig. 1: A two-year-old goat suffered from encephalitic listeriosis exhibiting unilateral facial paralysis with ipsilateral loss of corneal reflex dropped ear and protruded tongue.

outbreaks in Northern Oman from 2017 to 2019. No suspected cases were reported in other domestic or captive wild ruminants. The overall morbidity, mortality, and case fatality in goats were 5.8, 5.5 and 95%, respectively. While in sheep, the overall morbidity, mortality, and case fatality were 8.4, 4.2 and 50%, respectively. The overall prevalence of the disease was lower in the age group ≤12 months (5.9%) as compared to animals > 1-year-old (6.8%). While the case fatality was higher in animals ≤12 months (100%) as compared to animals > 1-year-old (50%) (Table 1).

Clinical signs: The constant clinical signs in all reported cases were fever, anorexia, moving aimlessly with a deviated neck, and circling. In addition, a loss of corneal reflex, dropping ear, protruded tongue, and hanging of jaw with drooling salivation ipsilateral to the lesion side were evident in some animals; mostly in animals > 1-year-old (Fig. 1). The reported clinical signs are summarized in (Table 2).

Serology: All sera were negative for CAE virus antibodies excluding the viral induced nervous manifestations in the examined cases.

Pathology: The pathology results are presented in Table 3 and Fig. 2. In most cases, no significant gross lesions were observed except slight meningeal congestion of the brain stem. All cases were negative for polioencephalomalacia where no cerebrocortical autofluorescent areas induced by thiamine deficiency were observed under ultraviolet light illumination during necropsy. The Gram staining of direct brain stem smears showed intra and extracellular Gram-positive rods. Microscopic lesions were found mainly in the hindbrain and, to a lesser extent, in the anterior part of the spinal cord. All cases exhibited diffuse microabscesses and perivascular cuffs in the pons, medulla oblongata and anterior spinal cord. The microabscess appeared as an area of liquefactive necrosis infiltrated with neutrophils. The perivascular cuffs consisted of macrophages, lymphoplasmocytic cells and a few neutrophils. There were neural degeneration and neurophagia in the inflammatory regions. Cerebellar and brain stem hemorrhages were also observed. Congestion and perivascular lymphoplasmacytic infiltrations were noticed in the meninges of the cerebellum, brain stem, and anterior spinal cord. The immunostaining of intracellular *L. monocytogens* antigens was visualized in the inflammatory cells in microabscesses, perivascular cuff, and glial foci.

Bacterial identification and antimicrobial sensitivity: Listeria species were isolated in pure cultures from all brain samples. The antimicrobial sensitivity of isolates A4 (Outbreak 7) and E3 (Outbreak 8), as well as the MARI of the 2 isolates, are presented in Table 4.

Table 4: Antimicrobial sensitivity antibiotics against *L. monocytogens* isolates from case 7 and case 8 and the calculated MARI for each isolate.

Antibiotic discs	Concentration (µg)	Sensitivity	
		Isolate A4 (Outbreak 7)	Isolate E3 (Outbreak 8)
Amoxicillin/clavulanic acid	30	++	+++
Amoxicillin	10	+	+
Ampicillin	2	+	+
Streptomycin	10	+++	+
Gentamycin	10	++	+
Sulfamethoxazole-trimethoprim	10	R	+
Ciprofloxacin	30	+	R
Enrofloxacin	5	R	R
Oxytetracycline	30	R	+
Tetracycline	30	R	+
Tylosin	15	R	R
MARI		0.5	0.3

R= resistant; MARI= Multiple Antimicrobial Resistance Indices

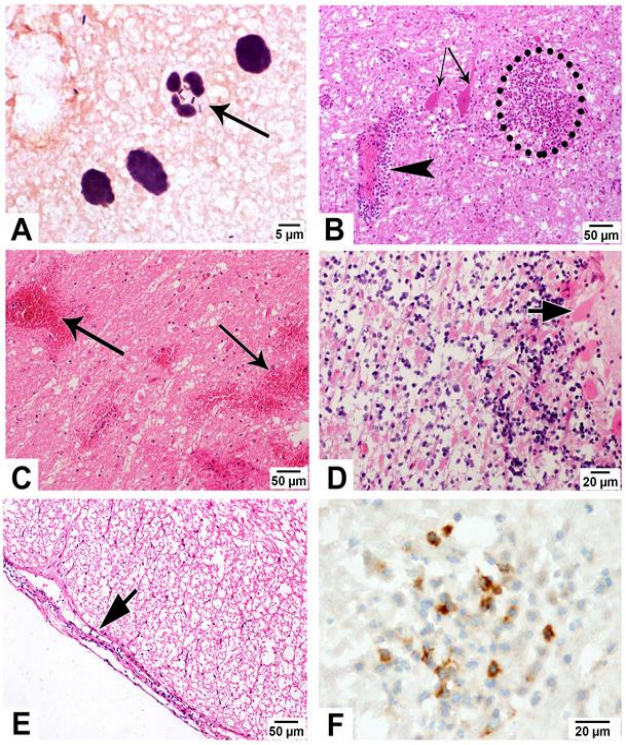

Fig. 2: Omani goat with encephalitic listeriosis (Case 6), hindbrain (brain stem and cerebellum), and anterior spinal cord. (A): Gram staining of the brain stem revealing intracellular Gram-positive rods within a neutrophil (arrow, scale bar = 5 µm). A-E: Histopathology sections stained with H&E. (B): Brain stem showing multifocal coalescing microabscesses (arrow) with neuronal degeneration (short arrow) and perivascular cuffing (arrowhead);) and (C) multifocal hemorrhages (arrows, scale bar = 50 µm). (D): Cerebellum showing degeneration of Purkinje cells with lymphoplasmacytic infiltrations (arrow; scale bar = 20 µm). (E): Anterior spinal cord showing meningeal lymphoplasmacytic infiltrations (arrow; scale bar = 50 µm). (F): Immunohistochemistry of brain stem showing positive intracellular immunostaining of L. monocytogenes in the area of microabscess (scale bar = 20 µm).

Molecular characterization: The partial 16S rRNA sequence (717 bp) of isolates A4 and E3 were submitted to the GenBank with accession numbers MW246569 and MW246570, respectively. The result retrieved from the GenBank indicated that our two sequences had a 98-99% similarity with *L. monocytogens*. Isolate A4 is closely associated with the isolates from food in different countries, while isolate E3 is closely related to the isolates from human clinical cases in other countries. In addition, the two isolates (MW246569 and MW246570) are closely related to the isolate from aborted cattle in the USA (Fig. 3).

DISCUSSION

Typical clinical signs and pathology of the encephalitic form of listeria infection, along with the positive results of bacteriology, immunohistochemistry, and molecular investigations of *L. monocytogenes* confirmed the occurrence of encephalitic listeriosis among small ruminants in the Sultanate of Oman.

Silage is feed storage and acid fermentation under anaerobic conditions (Elferink *et al.*, 2000). Growth of *Listeria spp.* in silage is usually associated with aerobic deterioration, causing severe risk to animal health (Fenlon, 1999). In Oman, small ruminants have cultural roots that are strongly linked to the region's ethnicity and are bred for their products as well as play an essential role in both intensive and semi-nomadic development systems (Payne, 1990). Moreover, it is widespread in Oman to feed small ruminants sun-dried sardines as a protein supplement since it is affordable and abundant (Mahgoub *et al.*, 2005). Silage has recently become more popular than the traditional sun-dried method in preparing fish meals for animal feed in Oman (Al-Abri, 2008; Al-Abri *et al.*, 2014). When pastures are scarce, leftover rotten bread and vegetables become an inevitable haven among some livestock breeders in urban areas (Barman *et al.*, 2020). Both fresh and decaying vegetables have been reported to be a source of *L. monocytogenes* (Ramaswamy *et al.*, 2007; Barman *et al.*, 2020). In the current study, all reported cases originated from Northern Oman near the Central Fruits and Vegetables Market in Muscat area. A history of feeding vegetable leftovers was reported in all the suspected cases. Unfortunately, we could not investigate the presence of *L. monocytogenes* in the suspected animal feed. *Listeria spp.* was detected in decaying vegetables in India, Malaysia, China, Chile, Uruguay, and other countries posing a public health risk (Fentahun and Fresebehat, 2012).

While listeriosis may be asymptomatic in carrier animals, it can be pathogenic in others and is known to occur in three clinical forms: encephalitic, septicemic, and intrauterine infections; and infrequently may cause mastitis, and keratoconjunctivitis (Fentahun and Fresebehat, 2012; Wei *et al.*, 2020). Our study's ovine and caprine cases showed typical clinical signs of the encephalitic form, including circling and deviated necks. Histologically, the microabscesses were evidenced by mononuclear and polymorph nuclear perivascular cuffing in the hindbrain. Positive listeria immunolabeling was detected in the microabscess and

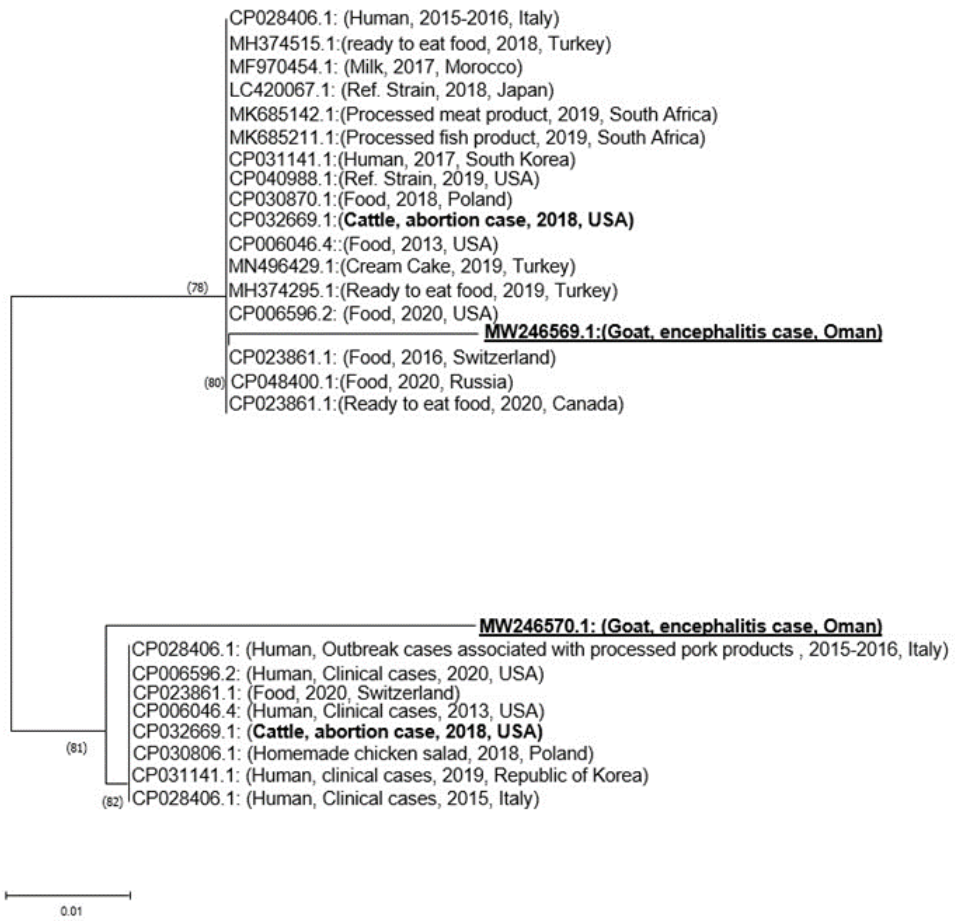

Fig. 3: Phylogenetic tree of the partial 16S rRNA gene sequences of two *L. monocytogenes* isolates (accession number MW246569 and MW246570) from the hindbrain of goats suffered from encephalitis in Oman. The scale bars represent 0.01.

perivascular cuffing aggregates of macrophages and neutrophils of the brain stem tissues in contrast to (Loeb, 2004), who detected positive staining in the microabscess but not in the perivascular cuffing. The clinical signs and the pathological findings of the encephalitic listeriosis observed in this study agreed with various outbreaks worldwide (Brugère-Picoux, 2008; Barman *et al.*, 2020).

The case fatality rates were higher in goats (95%) compared to sheep (50%), mainly due to the study's lower number of reported ovine cases. We could not retrieve any information about the treatment protocol for the registered infected farms following the infection; however, the recorded high fatality rates are probably due to the use of an antibiotic to which the listeria is resistant, in addition to the lower immunity of the affected animals.

We could not detect any pattern of infection that could be related to the weather. The study area is located on the coast and has a hot and humid climate most of the year (April- October), where the temperature and humidity subside from (November until March). In this study, all the cases were reported between July and November when temperatures ranged from 39°C to 26°C with rare heavy intermittent rainy days (Zurigat *et al.*, 2007). Although listeriosis has been reported to occur more frequently in the spring and winter (Fentahun and Fresebehat, 2012), our study showed that listeriosis can occur in hot and humid climates, not just in moderate to cold temperatures.

The antibiogram analysis varied between the two *L. monocytogenes* isolates from goat brain samples. Isolate A4 was utterly resistant to tetracycline, oxytetracycline, sulfamethoxazole-trimethoprim, enrofloxacin, and tylosin with a MARI of (0.5). While isolate E3 was resistant to ciprofloxacin, enrofloxacin, and tylosin with a MARI of (0.3). Listeria is a multidrug-resistant bacterium, and both isolates in our study had a MARI above the Krumperman permissible threshold of (>0.2), indicating a high risk/level of antimicrobial-resistant *L. monocytogenes*, which is alarming for both human and animal wellbeing (Woh *et al.*, 2023). Multi-drug resistant *L. monocytogenes* were isolated from encephalitic goats in India (resistant to doxycycline and erythromycin) (Barman *et al.*, 2020) and from milk samples of sheep and goats in Jordan (Obaidat and AlShehabat, 2023). The reported multidrug-resistant isolates in our study pose public health threats and could adversely affect the treatment scheme of listeriosis patients in Oman.

Although the gold standard for the diagnosis of listeriosis is the isolation of the bacteria from the infected animal tissues, the process is laborious and time-consuming. Molecular diagnostic tools, particularly the PCR are a more robust and quicker tool. In our study, we employed two universal primers (27F, 1492R) to amplify the 16S rRNA gene fragment, followed by sequencing and comparing with the available data in the GenBank. This enabled a more accurate diagnosis and thorough look into the gene structure of the isolates compared to similar isolates in the region or globally to help understand its epidemiology (Barman *et al.*, 2020). The current use of universal primers (27F/1492R) of the 16S rRNA locus is adequate for the identification of bacteria at the species level (Frank *et al.*, 2008), however, future studies should

warrant further utilization of more specific virulence genus levels, including *hly A* and *iap* genes (Kumar *et al.*, 2015).

Conclusions: The present study described outbreaks of fatal encephalitic listeriosis in goats and sheep between 2017 and 2019 fed on rotten vegetables and fruits from the central vegetable and fruit markets. This is the first report of multidrug-resistant *L. monocytogenes* isolates in the Sultanate of Oman. Such isolates pose public health threats and should be monitored to avoid spillover to humans. Further studies are required to assess the presence of the antimicrobial-resistant *L. monocytogenes* in fresh vegetables, fruits, and meat products and the surrounding environment, such as water and soil, through taking the One Health approach to determine the epidemiological status of listeriosis in Oman.

Author contributions: Conceptualization, MSE, HA; methodology, MES, HA, EIE, HGT; investigation MSE, RA, HA, KA, AA; resources, MSE, HA, EIE, AA; writing MES, HA, EIE, HGT; visualization, MES, HA, HCT, KA, AA; funding HA, EIE. All authors have read and agreed to the published version of the manuscript.

Ethical approval: No ethical approval was required from the Ethics Committee for Animal Use in Research, Sultan Qaboos University since tissue samples were obtained from dead animals or as part of a diagnostic investigation. However, informed consent was obtained from all animal owners.

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Conflicts of Interest: The authors declare no conflict of interest.

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