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Original article

DETECTION OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN DAIRY CATTLE FROM SHAHREKORD, IRAN

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Summary

Rahmani Dehkordi, M., Z. Hemati, M. Ghorbanpour, R. Goethe & L. Abdolmohammadi Khiav, 2023. Detection of *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle from Shahrekord, Iran. *Bulg. J. Vet. Med.* (online first).

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of an incurable intestinal disease in ruminants commonly known as Johne's disease (JD). This study aimed to evaluate the situation of MAP infection from dairy cattle by faecal PCR, milk PCR and milk culture in Shahrekord. In this study, from 19 herds, 80 individual milk and faecal samples were collected from cows of herds suspected to have JD. In addition, 19 tank milk and 19 corresponding environmental faecal samples from the milking parlour were collected. All samples were microscopically assessed; DNA were extracted using the phenol-chloroform method and analysed by IS*900*-PCR. Finally, milk samples were also cultured on Herold's Egg Yolk medium (HEYM). Based on microscopic results, eight individual milk (10%), five individual faecal (6.25%), five tank milk (26.3%) and three environmental faecal (15.8%) samples were positive for acid-fast bacteria. In IS*900*-PCR, MAP-DNA was detected in six individual milk (7.5%), one individual faecal (1.25%), two tank milk (10.5%) and two environmental faecal (10.5%) samples. MAP cultures were obtained on HEYM from three individual milk and three tank milk samples. Using this study design, six out of the 19 herds were identified as positive for JD. Furthermore, there wass a good agreement between ZN-staining and IS*900*-PCR (Kappa value 0.66). Sensitivity and negative predictive value of the IS*900*-PCR was determined as 100%. It can be concluded that serious precautions with respect to JD are needed in Shahrekord.

Key words: IS*900*-PCR, milk, *Mycobacterium avium* subsp *paratuberculosis*, Ziehl–Neelsen staining

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the cause of chronic, incurable intestinal disease in ruminants, commonly called paratuberculosis or Johne's disease (JD) (Gupta *et al.*, 2017; Biswal *et al*., 2018; Hemati *et al.*, 2020). JD causes severe economic losses due to the combination of reduction of milk production, weight loss, infertility, and control and prevention costs (Hemati *et al.*, 2019; Garvey, 2020). This disease has a wide global distribution, particularly in countries with dairy industry like Iran (Sechi & Dow, 2015; Singh *et al.*, 2016; Chaubey *et al.,* 2017). Some researchers believe that MAP is zoonotic based on the pathological similarities of JD and Crohn's disease (Sechi & Dow, 2015; Haghkhah *et al*., 2023). While there are several methods to detect MAP, their limitations may affect the results (Fernández-Silva *et al.*, 2011). Conventional faecal culture is generally suggested as a gold standard for the detection of JD. However, this method has several drawbacks including a long incubation time (up to 16 weeks) and low sensitivity (Collins, 1996). In addition, cultivation requires an external source of mycobactin J in the growth medium (Garvey, 2020). Nowadays molecular based methods have been performed to diagnose JD (Ssekitoleko *et al*., 2022). IS*900*-PCR assay as the most common MAP detection method, which shows better sensitivity than other diagnostic methods, can help for correct detection of animal samples (Singh *et al.*, 2020). Contamination of raw milk with MAP may also indicate an active infection. The presence of bacteria in raw milk increases concerns about the transmission of disease to people and the food chain.

JD is globally prevalent and has been widely reported in livestock species of Iran by various workers in different regions (Shrafati-chaleshtori *et al.*, 2009; Karimi *et al.*, 2012). Hence, JD control is essential to protect animal productivity and limit the spread of MAP to the human food chain. Therefore, the aim of this study was to evaluate the situation of MAP infection from dairy cattle by faecal-PCR, milk-PCR and milk-culture in Shahrekord, Iran.

MATERIALS AND METHODS

Sample collection

During this study, 19 cattle herds were randomly sampled between December 2021 and May 2022. The samples were randomly collected from different regions of Shahrekord province. Shahrekord is a province located in the southwest of Iran that is mountainous and has a moderate climate. At least 4 animals from each herd were sampled. Milk and faeces were collected from cattle suspected of having JD. A total of 80 individual milk samples and 80 individual faecal samples were obtained. To this end, animal udders were disinfected with 70% ethanol. Then, 40-45 mL of milk samples were collected in sterile screw-capped bottles. Samples of faeces were taken from the same animals. For a more detailed survey, 19 tank milk samples from the farms were taken after full milking, as well as 19 environmental faeces from the milking rooms. All samples were transported on ice directly to the bacteriology laboratory of the Faculty of Veterinary Medicine.

Ziehl-Neelsen staining

Ziehl-Neelsen-stained smear of faecal and milk samples were examined microscopically. Presence of pink coloured, acid-fast

short bacilli in bunch or irregular clusters were considered as MAP-positive (Hemati *et al.*, 2020).

DNA extraction

Isolation of DNA from raw milk was carried out as per Singh *et al.* (2019) with some modifications. Milk samples (40–45 mL) were centrifuged at 3,500 rpm for 45 minutes. After centrifugation, there were three distinct layers (top fat layer, middle whey layer and bottom sediment layer). The sediment was used for staining, culture and DNA extraction. For DNA isolation, 100 µL of lysis buffer (50 mM NaCL, 125 mM EDTA, 50 mM Tris-HCL; pH=7.6) were added to 500 µL of milk sediment and incubated at room temperature (RT) for 15 min. Then 100 µL of 24% sodium dodecyl sulfate (SDS) was added; incubated at RT for 10 min and heated at 80 ^{o}C for 10 min. Ten µL proteinase K (Sinaclon) was added and incubated at 55 ^{o}C for 1 hour. Then 115 µL of 5M NaCl and 93 µL CTAB-NaCl was added, and incubated at 65 ^{o}C for 30 min. Equal volume of phenol:chloroform:isoamyl (PCI) alcohol (25:24:1) was added and centrifuged at 12,000 rpm for 5 min. The aqueous phase was transferred to Eppendorf tube to precipitate DNA by adding 0.8 volumes of chilled isopropanol. The tube was kept at 20 ^{o}C overnight, then centrifuged at 12,000 rpm, for 10 min at 4 ^{o}C and the supernatant was discarded. The pellet was mixed with 1000 µL of 70% ethanol by pipetting and centrifuged at 12,000 rpm for 1 min, the supernatant was then decanted. The remaining pellet was re-suspended in 35 µL TE buffer and stored at –20 ^{o}C until use.

For DNA extraction, faecal samples (6–8 pellets) were obtained with gloved hands directly from the animal rectum (Van Embden *et al.*, 1993). In the laboratory, about 2 g faecal sample was crushed in sterile pestle mortar with 10 mL of 1× PBS to make a fine paste. The mixture was allowed to stand for 2–5 min at RT, and then was poured on tube. Tubes were centrifuged at 3,500 rpm for 40 min. After centrifugation three layers were visible. The upper crust was removed and the middle semi-solid layer was used for staining and extraction of DNA. For the isolation of DNA, the semisolid intermediate layer was taken with a sterilised swab and suspended in 500 µL of sterilised 1× PBS. Each sample was heated at 95 ^{o}C for 10 min, cooled and 50 µL lysozyme was added to each sample. Sample was incubated in 37 ^{o}C for 60 min; 50 µL of SDS 20% and 10 µL proteinase K (Sinaclon) were added and incubated at 55 ^{o}C for 60 min. Then 115 µL NaCl (5M) and 93 µL CTAB were added and incubated at 65 ^{o}C for 30 min. The samples were mixed with equal volume of PCI (25:24:1) and centrifuged (12000 rpm, 5 min). Then DNA was precipitated by adding 0.8 volume isopropanol and incubated overnight at –20 ^{o}C. After centrifugation, 70% ethanol was added and centrifuged again (12,000 rpm, 5 min). The supernatant was then decanted and the remaining pellet re-suspended in 35 µL TE buffer and stored at –20 ^{o}C until use.

IS900-PCR for MAP infection diagnosis

The isolated DNA from each milk and faecal sample was subjected to IS*900*-PCR using MAP specific primers (P90: 5' GAA GGG TGT TCG GGG CCG TCG CTT AGG 3'; P91: 5' GGC GTT GAG GTC GAT CGC CCA CGT GAC 3') targeting the IS*900* element, a unique MAP insertion sequence according to Millar *et al*. (1996). In brief, 5 µL of faecal and milk DNA was added to 20 µL of the PCR mixture (1 µL P90 and P91 or 10

pmol/μL, 5.4 μL 10× PCR-buffer, 2.5 μL dNTP, 9.1 μL H_2O, and 1 μL *Taq* polymerase: 5 U/μL). Samples were subjected to an initial denaturation step (95 °C for 5 min), 35 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 30 s and extension at 72 °C for 30 s. A final extension was performed at 72 °C for 7 min. A negative control (sterile water), and a positive control containing DNA from reference strain as template, were included. Samples yielding a positive amplification for the product of 413 bps were found to be positive for MAP bacilli.

Milk culture

Following centrifugation of all individual and tank milk samples, the sediment layer was collected and used for the MAP culture. The sediment layer was transferred to a sterilised tube containing 40 mL of 0.9% hexadecyl pyridinium chloride (HPC). The tubes were allowed to stand for 18–24 hours at RT. After decontamination, supernatant was carefully discarded and nearly 1 mL of sediment was inoculated into 1 tubes of Herrold's egg yolk medium (HEYM) with mycobactin J and 1 tube without mycobactin J. Tubes were incubated at 37 °C for 3–4 months and checked for growth every 7 days. Colony morphology and mycobactin dependency was checked. All grown colonies were confirmed as MAP by ZN-staining and IS*900*-PCR test

Data analysis

After the registration of the results, the overall prevalence of Johne's disease in the industrial cattle farms of Shahrekord city was calculated. The results were analysed by contingency tables and compared using the McNemar exact test using SPSS software (version 16.0, SPSS Inc., Chicago, USA).

RESULTS

ZN-test results of 198 samples were collected. In total, 21 (10.6%) of the 198 samples tested positive (ZN positive) (Table 1). Eight (10%), five (6.25%), five (26.3%) and three (15.8%) of milk, stool, bulk milk or environmental stools samples, respectively, were found positive. Positive stool and milk samples contaminated with MAP bacteria were observed as shown in Fig. 1.

IS*900*-PCR results of 198 samples are given separately in Table 2. Six (7.5%),

Table 1. Results of microscopic examination of ZN-stained smears of 198 cattle in Shahrekord, suspected of clinical JD

Microscopy	NEG	1+	2+	3+	4+	Total (P)
Milk samples	72 (36.3 %)	3	3	2	0	8 (10 %)
Faecal samples	75 (37.9 %)	0	5	0	0	5 (6.25 %)
Tank milk samples	14 (7.1 %)	1	1	1	2	5 (26.3 %)
Faeces from the milking rooms	16 (8.1 %)	0	2	0	1	3 (15.8 %)
Total	177 (89.4 %)	4 (2.02 %)	11 (5.5 %)	3 (1.5 %)	3 1.5 %)	21 (10.6 %)

NEG (absent of bacilli in 10 fields), 1+ (10 bacilli or one bunch), 2+ (10 bacilli or one bunch in alternate 2, 3 or 4 fields), 3+ (10 bacilli or one bunch in alternate fields, 4+ (10 bacilli or one bunch in each field).

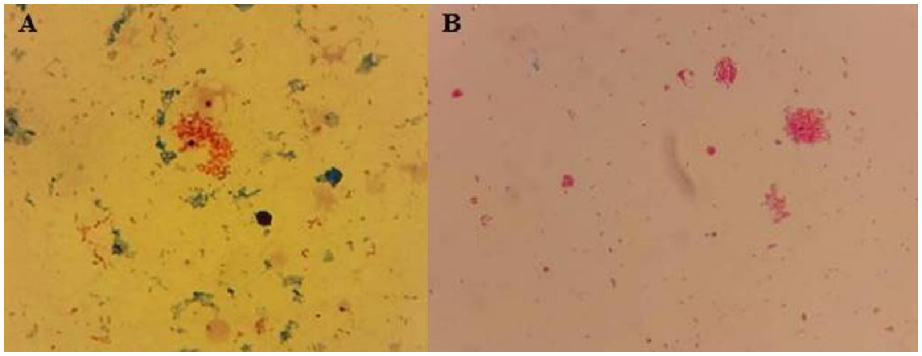

Fig. 1. Microscopic view of acid fast bacilli under oil immersion (100×).
A. Representative faecal samples; **B.** Representative milk samples.

Table 2. Results of IS*900*-PCR examination of 198 cattle in Shahrekord, suspected of clinical JD

IS-*900*	NEG	Positive	Total
Milk samples	74 (92.5 %)	6 (7.5 %)	80
Faecal samples	79 (98.75 %)	1 (1.25 %)	80
Tank Milk samples	17 (89.5 %)	2 (10.5 %)	19
Faeces from the milking rooms	17 (8 %)	2 (10.5 %)	19
Total	187 (94.5%)	11 (5.5 %)	198

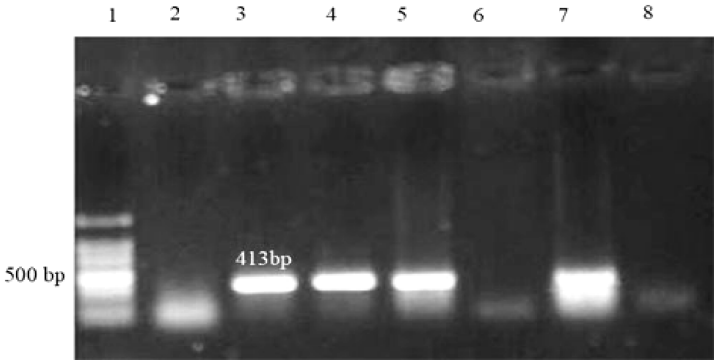

Fig. 2. IS*900*-PCR results. Lane 1: DNA ladder; lane 2: negative control; lane 3: positive control; lanes 4, 5, 7: representative milk and faecal samples contaminated with MAP; lanes 6, 8: representative milk and faecal samples without contamination with MAP.

one (1.25%), two (10.5%) and two (10.5%) of milk, faecal, tank milk and environmental faecal samples, respectively, tested positive. In total, 11 (5.5%) of the 198 samples tested positive by PCR (Table 2 and Fig. 2).

Totally, three milk and three tank samples were MAP positive after culture on Herold's Egg York medium (HEYM) medium. Small, smooth, cream-coloured, semi-transparent, hemispherical, and almost shiny colonies were observed only in

Fig. 3. Colonies of MAP after 15 weeks of culture on a HEYM with mycobactin J (left) and without mycobactin J (right).

HEYM with mycobactin J (Fig. 3). Subsequently, all grown colonies were confirmed as MAP by ZN-staining and IS*900*-PCR (Fig. 4). Bacterial cells from MAP colonies were strongly acid-fast and appeared pink in ZN staining. They generally appeared in clumps.

This study showed a good agreement (Table 3) between ZN-staining and IS*900*-PCR (Kappa value 0.66; $P<0.05$). Sensitivity and negative predictive value of the IS*900*-PCR were both 100%.

Table 3. The status of MAP infection in cattle by screening of ZN-staining and IS*900*-PCR ZN-staining

Count		IS*900*-PCR		Total
		–	+	
ZN-staining	–	177	0	177
	+	10	11	21
Total		187	11	198

DISCUSSION

Johne's disease caused by MAP leads to diarrhoea, progressive emaciation and reduction in milk production (Ahlstrom *et al.*, 2015; Hemati *et al.*, 2020; Wright *et al.*, 2022; Haghkhah *et al*., 2023). JD occurs in domestic and wild ruminants all over the world and causes significant economic losses to the livestock industry (Biswal *et al*., 2018; Whittington *et al.*, 2019). Various diagnostic methods have been used to identify the MAP. Because JD has slow progression and also delayed immune response, it is suggested to use a combination of diagnostic tests to identify MAP (Slana *et al.*, 2008). The most common methods of JD diagnosis are cultivation, microscopic examination, PCR and ELISA, but each of these methods has drawbacks (Fecteau, 2018). In this study, the microscopic examination showed that eight (10%) milk samples, five (6.25%) faecal samples, five (26.3%) tank milk

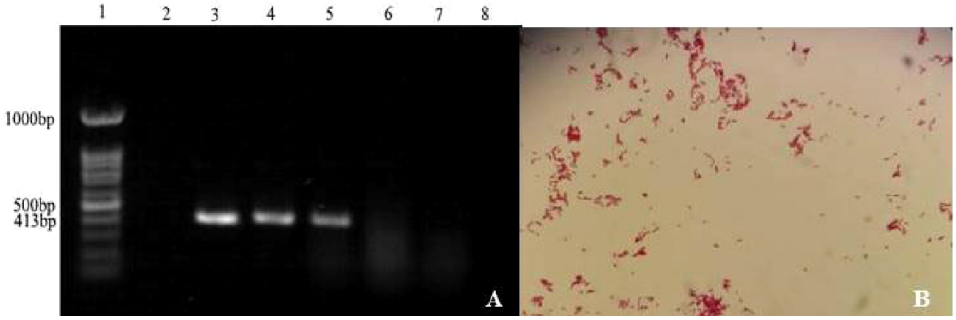

Fig. 4. Confirmation of the grown colonies: **A.** Colony-PCR test. Lane 1: DNA ladder; lane 2: negative control; lanes 3, 4, 5: milk samples. **B.** ZN-stained colonies.

and three environmental faeces (15.8%) were positive for acid-fast bacteria. In PCR, six (7.5%) milk samples, one (1.25%) faecal sample, two tank milk (10.5%) and two (10.5%) environmental faeces were positive for MAP. All the eleven samples that were positive in PCR were also positive in the microscopic examination. Microscopic results point only to the presence of mycobacteria, i.e. acid-fast bacteria with a characteristic shape. Further differentiation is not possible. Because many other mycobacteria species can be found in the environment and can be introduced in milk samples and faeces from the milking rooms by contamination, it is not justified to assume that all microscopically positive samples contain MAP (Huntley *et al.*, 2005). Based on our results, only six samples grew on HEYM. MAP has a long incubation period (up to 16 weeks) and the decontamination procedure adversely affects the viability of bacteria in culture media (Singh *et al.*, 2020). Furthermore, this method can only detect living bacteria while other methods such as ZN staining and molecular methods detect living and dead bacteria (Kralik *et al.*, 2011). Additionally, in our study, six of the 19 suspect herds tested positive for JD by at least one method. There was an agreement between the PCR results and the culture results, respectively, of individual milk samples of animals from two herds and of the corresponding tank milk sample. Comparison of three antigen detection tests for detecting contamination with MAP in different samples and farms is presented in Table 4.

In 2004, Anzabi and colleagues evaluated the contamination of cattle milk with MAP in Tabriz, another province in Iran.

Table 4. Comparison of three antigen detection tests for detecting contamination with MAP in different samples and farms

	Samples	Farm name	Microscopy	Culture	IS*900*/413
1	Milk sample No. 3	A	1+	N	P
2	Milk sample No. 4	A	3+	P	P
3	Tank milk samples	A	4+	P	P
4	Faeces from the milking rooms	A	2+	N	P
5	Milk sample No. 6	K	1+	P	N
6	Faecal sample No. 6	K	2+	N	N
7	Faeces from the milking rooms	K	4+	N	P
8	Milk sample No. 11	L	2+	P	P
9	Milk sample No. 14	L	1+	N	P
10	Faecal sample No. 14	L	2+	N	P
11	Tank milk samples	L	4+	P	P
12	Milk sample No. 22	M	2+	N	N
13	Tank milk samples	M	2+	N	N
14	Milk sample No.. 41	O	3+	N	P
15	Milk sample No. 42	O	2+	P	P
16	Faecal sample No. 41	O	2+	N	N
17	Tank milk samples	O	1+	N	N
18	Faeces from the milking rooms	O	2+	N	N
19	Faecal sample No. 56	R	2+	N	N
20	Faecal sample No. 75	R	2+	N	N
21	Tank milk samples	R	3+	N	N

N: Negative, P: Positive

In their study, milk samples were collected from 80 healthy cows and 80 cows from 19 herds suspected of having JD, plus 20 pasteurised milk samples, and 20 milk samples tested for MAP. Their results showed 17 (21.25%) and 25 (31.25%) cases from suspicious cattle were positive by culture and PCR, respectively. Six (7.5%) and 14 (17.5%) cases from healthy cattle were positive for culture and PCR, respectively (Anzaby *et al.*, 2006). The rate of MAP infection in their study was slightly higher than our study findings.

In another study, 338 serum and faecal samples from cows over 18 months of age were obtained from 14 dairy farms in Tehran province, Iran. MAP contamination in cattle was estimated to be 3.6% and 9.5% using culture and ELISA (Heidarnejhad *et al.*, 2017). In addition, the JD prevalence in the flock was calculated at 28.6% by cultivation and at 57.14% by ELISA. In our study, 3 out of 19 flocks (15.79%) were positive for MAP with positive culture, which was lower than the results of Heydaranjad *et al.* (2017). The low prevalence of MAP in this study may be due that we have cultured milk samples and the cited study: faeces.

Karimi *et al.* (2012) reported that the rate of contamination of bovine tissue samples by MAP at Shahrekord was 6.67% and 26.67% with ZN and PCR, respectively. In another study, a total of 373 bovine faeces, 150 cow milks and 73 bulk milks were randomly selected. Based on qPCR results, MAP-DNA was detected in 68.66% (103/150) of the cow faeces, 12% (18/150) of individual cow milk samples and 52.05% (38/73) of tank milk samples (Hanifian *et al.*, 2013). The rate of MAP infection in individual milk and bulk tank milk samples was slightly higher than findings of our study. The high prevalence of MAP in this study could be attributed to the size of the farm, the number of animals on the farm, the increase in the number of samples and sanitary conditions. In 2016, Soltani evaluated the MAP contamination in individual cattle milk in Kerman, Iran and showed that 15.1%, 20.7% and 24.52% of milk samples were positive for MAP in culture, PCR and nested PCR, respectively (Soltani, 2018).

The prevalence of infection on herd level in different countries is as follows: In India, MAP contamination in cattle was 23.25% and 58% using PCR and culture, respectively (Shankar *et al.*, 2010). MAP contamination in the faeces of dairy cattle in Nepal was reported to be 13.57% and 16.59% with culture and PCR, respectively (Singh *et al.*, 2020). In Iraq, MAP contamination in individual cattle milk was 6% using PCR (Ahmed *et al.*, 2020).

Based on the previous findings, the prevalence of infection in different geographical locations in Iran is reported as follows: Yasouj 1.5% (Pourjafar & Badiei, 2005), Ahwaz 2% (Haji Hajikolaei *et al.*, 2006), Mazandaran 5.6% (Sadati *et al.*, 2012), Uremia 12% (Dilmaghani *et al.*, 2011), Chaharmahal and Bakhtiari 3% (Shrafati-chaleshtori *et al.*, 2009), and Markazi 15.5% (Ghaem *et al.*, 2012). In this study, 5.5% of dairy cattle were MAP positive by PCR, which is similar to the study in Chaharmahal and Bakhtiari province (Karimi *et al.*, 2012). In this study, the sensitivity and negative predictive value of IS*900*-PCR were calculated at 100% as in other studies (Huntley *et al.*, 2005). Today, PCR has been widely used to detect MAP. However, disadvantages for the method include the quality of the genomic DNA and the presence of inhibitors such as bile salts, bilirubin, and urobilinogen in stool samples which may affect the results. On the other hand, the PCR assay is simple and can be done within 24

hours; it also can detect dead bacteria. The PCR test greatly reduces costs compared to conventional methods (Khare *et al.*, 2004). In this study, the combination of Ziehl-Neelsen staining and IS*900*-PCR detected 16.2% (32/198) of faecal and milk samples as positive for MAP infection. According to this result, the combination of methods led to an increase of sensitivity, similar to other studies *(*Juste *et al.,* 2005*;* Pinedo *et al.*, 2008).

CONCLUSIONS

The results of this study demonstrated the presence of MAP in the milk and faeces from infected cattle and may be an indicator for infection and active contamination in Shahrekord livestock. In addition, MAP was detected from tank milk and environmental faeces, so it can be concluded that excretion of bacteria through faeces and milk causes environmental contamination. The presence of bacteria in raw milk increases concerns about the transmission of disease to people and the food chain. The findings of this study highlight the need for JD prevention policies in this geographic area.

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