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Isolation and Identification of *Mycobacterium avium* Subsp. *paratuberculosis* from Milk, Manure and Fecal Samples of Holstein-Friesian Dairy Cattle

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Abstract: The main aim of this study was detection of subclinical forms of Johne's disease on dairy farms which were suspected of having Johne's disease or history of it and confirmatory paraclinical of paratuberculosis in them. Also, the isolated bacteria will be used for genotyping and molecular epidemiology of paratuberculosis in the next studies. Samples were gathered of 137 farms in different parts of Razavi Khorasan Province of Iran. There were 505 fresh-fecal and manure samples and 148 raw milk samples including the bulk milk transporters, milk samples of cows with paratuberculosis clinical signs and the farm bulk milk tanks. The samples were cultured on Herrold's egg yolk medium with and without mycobactin J. The samples, in which the bacteria could merely grow on Herrold's media containing mycobactin J, were considered to be positive. Additional tests such as colony morphology, acid fast staining and Nested PCR-IS900 assay were performed to confirm isolated bacteria. Diagnostic test of *M. paratuberculosis* culture was positive in milk, manure and fresh-fecal samples of 29 farms of 137 farms. Out of 653 samples, 50 cases have found positive after cultivation. The number of the bacterial colonies was varied from 1 up to 250. In addition to fresh-fecal samples, using of manure samples and milk samples taken from farm bulk milk tanks can help to detect subclinical forms of Johne's disease and disease screening in dairy farms. For control of Johne's disease, mainly subclinical or hidden forms are noticed. In order to achieve this aim, the presences of *Mycobacterium avium* subsp. *Paratuberculosis* were investigated in different samples.

Key words: *Mycobacterium avium* subsp. *paratuberculosis,* milk, fecal, manure, Holstein-Friesian

INTRODUCTION

Mycobacterium paratuberculosis causes paratuberculosis, commonly known as Johne's Disease (JD) (Grant *et al*., 1998). Paratuberculosis is a chronic granulomatous enteritis that affects domestic and wild ruminants. Johne's disease was reported first in 1957 in Jersey and Sindhi cattle of National Oil Company farm of Abadan in Southern Iran (Baharsefat *et al*., 1972). Because of its long incubation period, mainly subclinical or hidden forms of Johne's disease are noticed. However, after a long subclinical phase, clinical signs can be recorded, such as cachexia and diarrhea (Bhidea *et al*., 2006). Currently, no country in the world can claim that it is free from this disease. Nowadays, not only economic effects but also zoonotic aspects of JD have very specifically taken the attention of mycobacteriologists (Bazargani *et al*., 2007). Paratuberculosis caused by a small, gram-positive, non-motile, non-sporing, acid-fast and facultative anaerobic intracellular bacterium. However, some non-acid fast, lightly acid-fast and cell wall-deficient types are encountered. The bacilli generally occur in clumps linked together by a network of intercellular filaments (Ayele *et al*., 2001; Caldow and Gunn, 2003). It grows slowly and is a non-chromogenic, biochemically, unreactive organism that requires mycobactin-enriched media for growth. *M. paratuberculosis* contains approximately 18 copies of the insertion sequence IS900. Because this repetitive element is considered specific for *M. paratuberculosis* organisms, it has been used in the development of DNA-based diagnostics (Cousins *et al*., 2000). On Herrold's Egg Yolk Agar Medium (HEYM), one of the most commonly used culture media in veterinary diagnostic laboratories, the colonies appear small, somewhat rough and off-white to yellow in color (Collins, 2003). *M. paratuberculosis* strains have been classified into three groups (types I, II and III) based on culture characteristics and molecular characterization by PFGE and IS900-RFLP (De Juan *et al*., 2006). Subclinically or clinically infected animals shed

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M. paratuberculosis in feces and milk, enabling dissemination to susceptible calves, the environment and in retail milk (Buergelt and Williams, 2004; Dundee *et al*., 2001; Radostits *et al*., 2007). The successful isolation of *M. paratuberculosis* from raw milk depends on decontamination methods, the age of raw milk and temperature of storage until the time of culture. According to some reports, the isolation of *M. paratuberculosis* from milk samples older than 8 days is almost impossible (Gao *et al*., 2005; Radostits *et al*., 2007). Fecal culture is presently recognized as the most reliable index of infection in live cattle. A major advantage of fecal culture is that it can identify cattle 1-3 years prior to the appearance of clinical signs (Radostits *et al*., 2007). Fecal culture by radiometric technique is also available. This method is faster and has slightly higher sensitive than conventional culture systems but is more expensive and requires specific instruments. PCR assays are less sensitive than fecal culture for detection of small numbers of bacteria, perhaps due to inhibitory substances in feces (Gumber and Whittington, 2007; Radostits *et al*., 2007; Songer and Post, 2005). Culture also is an essential step for later application of the standardized molecular typing techniques IS900-RFLP (De Juan *et al*., 2006; OIE, 2004; Stabel *et al*., 2002).

The main aims of this study were detection of subclinical forms of Johne's disease on dairy farms which were suspected of having Johne's disease or history of it and confirmatory paraclinical of paratuberculosis in them. Also, the isolated bacteria will be used for genotyping and molecular epidemiology of paratuberculosis in the next studies.

MATERIALS AND METHODS

From the middle of May 2006 to late October 2007, a total of 505 fresh-fecal and cattle manure samples and 148 raw milk samples including the bulk milk transporters, milk samples of cows with paratuberculosis clinical signs and the farm bulk milk tanks were taken from 137 dairy farms from different parts in Razavi Khorasan Province, Iran. With the exception of milk samples of the bulk milk transporters, other milk samples were taken from both cows infected by the clinical form of paratuberculosis and bulk milk tank of farms whose fecal samples were also taken. None of manure samples were taken from farms whose milk or fecal samples were collected. Also, none of the farms were bacteriological tested for detection of *M. paratuberculosis* before the study.

Medium preparation: For cultivation of *M. paratuberculosis* Herrold's medium was prepared according to World Organization for Animal Health recommendation (OIE, 2004). In order to prevent bacterial and fungal contamination, 50 mg L^{-1} nalidixic acid, 50 mg L^{-1} amphotericin B and 50 mg L^{-1} vancomycin were used as specific antibiotics.

Fresh fecal samples: A total of 379 fecal samples were collected from dairy farms. The samples were directly taken from the cows' rectum. Of the fecal samples, 16 samples were taken from cows with advanced clinical signs of Johne's disease. Out of 363 remaining samples, 53 were taken from cows without clinical signs but having contact with clinical cases of Johne's disease and the rest of the samples had no contact with diseased cattle.

Following collection, samples were immediately placed into a container with ice packs and transported to the laboratory. Ten gram of each sample was stored at -70°C until bacterial culture (for about 3 months).

Manure samples: A total of 126 bovine manure samples were taken from different farms. These farms had history of Johne's disease. Of these samples, 63 samples were taken from outdoor and 63 samples from the same frames from indoors. Following collection, all samples were transported to the laboratory under cold condition and stored as above.

Raw milk samples: The raw milk samples (148) were obtained from different regions. Out of these numbers, 67 samples were from bulk milk transporters with the capacity of 10000 up to 15000 L. In this manner 69 samples taken from the bulk milk tank of farms which were suspected of having Johne's disease and 12 samples from cows which showing clinical signs of Johne's disease. Following collection, all samples were transported to the laboratory under cold condition. Appropriate amount of milk sample (50 mL) was centrifuged at 4°C (3100 g, 15 min). Then cream and pellet fractions were mixed together and kept at -70°C for about 2 months until the time for bacterial culture.

Fresh-fecal and manure culture: According to OIE recommendations, 1 g of each fecal sample was transferred to a 50 mL tube containing 20 mL of sterile distilled water and placed on a shaker for 30 min at room temperature. The larger particles were allowed to settle for 30 min. The upper phase (5 mL) was transferred to a 50 mL tube containing 20 mL of 0.75% hexadecylpyridinium chloride (HPC) for decontamination. The tubes were inverted several times to assure uniform distribution and allowed to stand undisturbed for 18-20 h at room temperature. Hundred to 200 μL of the undisturbed sediment was transferred to slant (four test at time) HEYM

medium containing antibiotic (50 µg mL^{-1} nalidixic acid, 50 µg mL^{-1} amphotericin B and 50 µg mL^{-1} vancomycin), three slants with mycobactin J and one slant without mycobactin J. The slants were incubated at 37°C for 20 weeks and observed weekly from the sixth week onwards. The samples, in which the bacteria could merely grow on Herrold's media containing mycobactin J, were considered to be positive. Additional tests such as colony morphology, acid fast staining and Nested PCR-IS900 assay were performed to confirm of isolated bacteria.

Milk culture: The isolation was done as reported previously by Dundee *et al*. (2001), Gao *et al*. (2005) and Grant *et al*. (2005, 2001). With some modifications, briefly, each pellet and cream from the centrifugation of the last stage, were re-suspended in 20 mL of HPC 0.75% (w/v) and incubated for 5 h at room temperature. Following incubation, the samples were centrifuged at 4°C (3100 g, 15 min). Then the pellet was separated from supernatant and resuspended in 1 mL sterile PBS buffer. The new suspension (100-200 µL) was used for transferring on HEYM media, similar to fecal culture method.

Colonial morphology study: The isolate bacteria were examined by colonial morphology study and observation as well as acid fast staining.

DNA extraction: DNA extraction was performed by taking a few of single colonies. The single bacterial colony was taken from any HEYM medium containing mycobactin J and re-suspended in 50 µL distilled water in a screw-cap micro-centrifuge tube. The samples were boiled for 20 min prior to being centrifuged for 5 min /14000 g to settle cell debris. Two microliter of supernatant, containing the genomic DNA, was used for Nested PCR-IS900 amplification (Hosek *et al*., 2006).

Nested PCR: PCR assay was performed for confirmation of *M. paratuberculosis* and was done according to report Erume *et al*. (2001) with some modifications. Amplification was carried out in a 50 µL final volume. This consisted of a 5 µL DNA sample mixed with a 45 µL master mix containing 67 mM Tris-HCl pH 8.8, 2 mM $MgCl_2$, the four deoxyribonuleotide triphosphates, dATP dCTP Dgtp dTTP (100 µM each), 1 µM of each of the oligonuleotide primers, 2.5 U *Tag* polymerase in a 0.5 mL Eppendorf tube. Samples were subjected to an initial denaturation step of 94°C for 5 min and then to 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min in a Eppendorf Master cycler gradient. DNA of *M. paratuberculosis* strain 316F and sterile buffer were used as positive and negative controls, respectively.

Nested PCR assay was done with primers para 1 (5'-TGA TCT GGA CAA TGA CGG TTA CGG A -3') and para 4 (5'- CGC GGC ACG GCT CTT GTT- 3') that were used to amplify a 563 bp target region of *M. avium* subsp. *paratuberculosis* IS900 sequence. Then, 5 µL of each of the initial amplification products was transferred to new tubes containing the same reaction mixture described above (exception of oligonucleotide primers para 1 and para 4) and re-amplified using the primers para 2 (5'- GCC GCG CTG CTG GAG TTG A -3') and para 3 (5'- AGC GTC TTT GGC GTC GGT CTT G -3').

Agarose gel electrophoresis: The second PCR products were analyzed on a 1.2% agarose gel containing 0.5% ethidium bromide and visualized by UV-light transilluminator.

RESULTS AND DISCUSSION

In this study, we identified subclinical forms of Johne's disease and dairy farms which were suspected of having Johne's disease in Razavi Khorasan Province, Iran. Razavi Khorasan Province is one of the largest provinces in Iran, in terms of size and animal populations. That is located in the north east of Iran and borders with countries of Afghanistan and Turkmenistan. Diagnostic test of *M. paratuberculosis* culture was positive in milk, manure and fresh-fecal samples of 29 of 137 farms examined.

Colonial morphology: It was possible to observe *M. paratuberculosis* colonies on Herrold media containing mycobactin J from the 9th week onward. In the beginning, colonies were small, so, that they appeared about 1 mm in diameter, but their diameter gradually increased up to 2-3 mm. The colonies were observed spherical, semi-transparent or translucent, bright in surface, smooth to somewhat rough and off-white to yellow in color (Fig. 1). The number of bacterial colonies was variable from 1 up to 250 colonies. The most and the least number of colonies were found in the fresh-fecal samples of clinically affected cows and raw milk samples of farm bulk milk tanks, respectively (Table 1). All the isolated bacteria were confirmed with colony morphology, acid fast staining and Nested PCR-IS900 assay.

Nested PCR: All isolated strains were PCR positive and target gene with 210 bp size were observed, which is corresponded with size of band of positive control (*M. avium* subsp. *paratuberculosis* strain 316F) (Fig. 2).

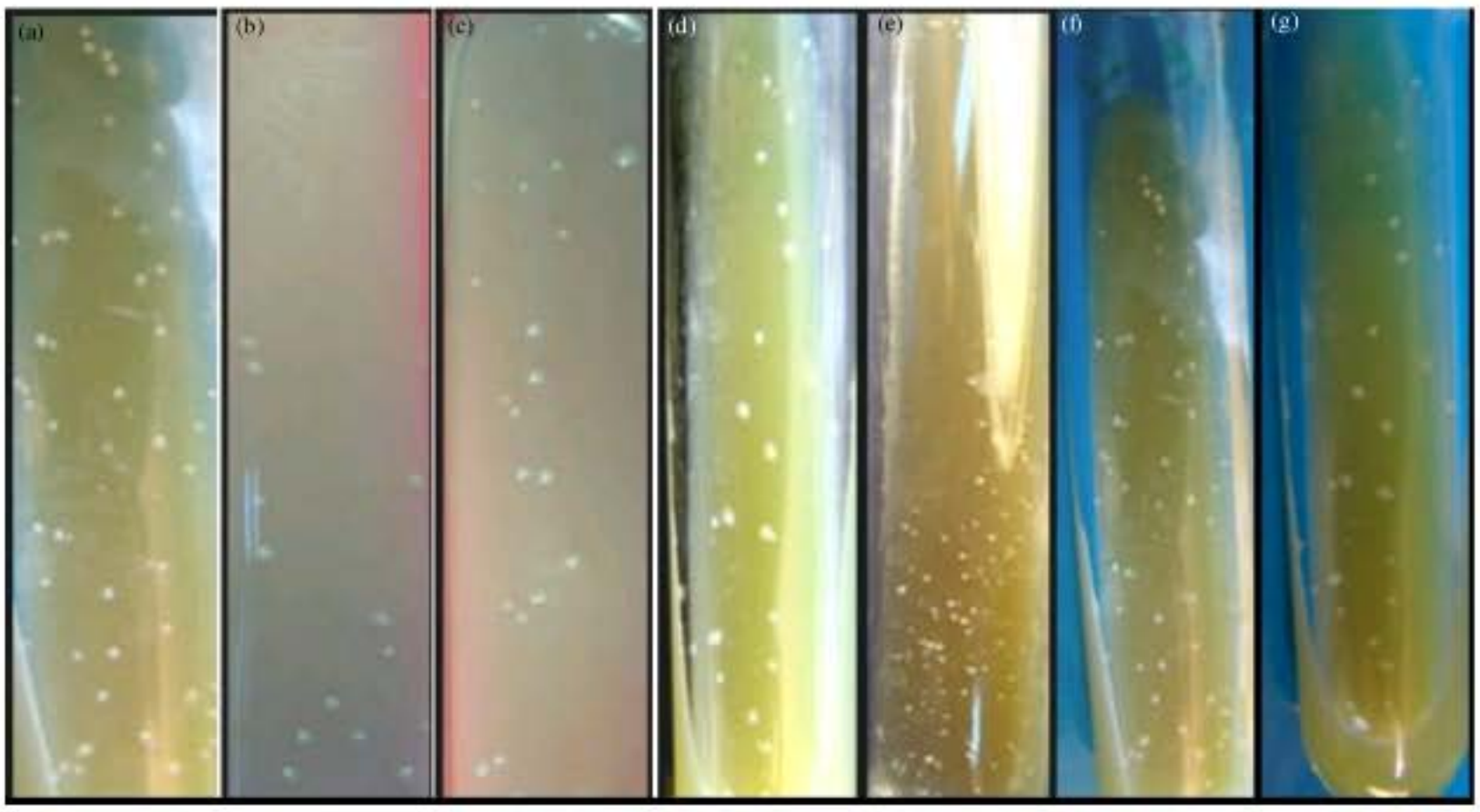

Fig. 1: *Mycobacterium avium* subsp. *paratuberculosis* colonies showing on Herrold's egg yolk medium containing mycobactin J. (e) Colonies isolated from cows showing clinical signs of Johne's disease, (a-c) colonies isolated from raw milk samples and (d, f and g) bacteria isolated from fecal samples of the cows without the sings of Johne's disease

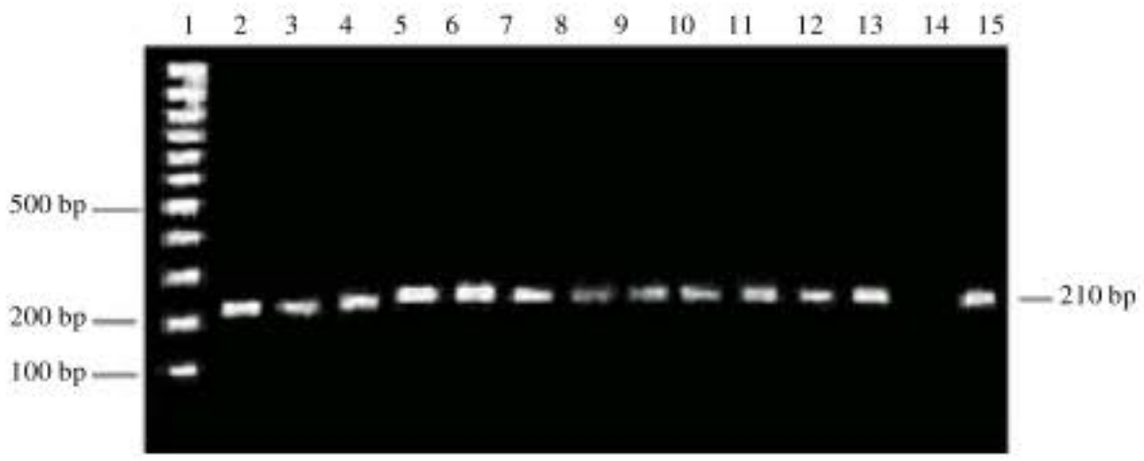

Fig. 2: The result of Nested-PCR of *M. avium* subsp. *paratuberculosis* isolated from fecal and milk samples growth in specific media. Lane 1 molecular weight standard (100 bp), lane 2-13 isolated strains, lane 14 negative control (sterile buffer), lane 15 positive control (*M. avium* subsp. *paratuberculosis strain* 316F)

Table 1: The number of positive tubes and colonies related of milk, manure and fecal culture

	Raw milk (total of positive tubes)			Feces (total of positive tubes)				
Colony count	Cows with clinical signs	Farm bulk milk tanks	Bulk milk transporters	Cows with clinical signs	Cows without clinical signs (contact)	Cows without clinical signs (Non-contact)	Outdoors	Indoors
1-10	0	5	0	0	0	0	0	0
11-50	10	0	0	0	11	4	3	7
51-100	1	0	0	21	0	0	0	0
101-250	0	0	0	12	0	0	0	0
Total	11	5	0	33	11	4	3	7

Isolation of *M. paratuberculosis* from manure and feces: Out of 16 samples taken from the cows with clinical signs, 13 cases (81.3%) have been found positive after cultivation, while this number for the 363 cows without clinical signs of Johne's disease were 15 (4.1%) (Table 2). As mentioned, the cows without clinical signs are divided into two groups. Out of 53 cows which had contact with clinically cases of Johne's disease, 11 of them were positive (20.8%), while this value for another group was 1.3% (4 samples from 310 remaining samples). As parallel to progress of the disease, switching from subclinical to clinical, bacterial shedding rate in milk and especially in

Table 2: Fresh fecal culture results for cows showing clinical signs of Johne's disease and cows without clinical signs which have contacts with clinically cases of Johne's disease and those which have none contact with them

	Positive		Negative		Total	
Fresh fecal samples	Count	Row (%)	Count	Row (%)	Count	Total (%)
Cows with clinical signs	13	81.3	3	18.7	16	100.0
Cows without clinical signs (contact)	11	20.8	42	79.2	53	100.0
Cows without clinical signs (Non-contact)	4	1.3	306	98.7	310	100.0
Total	28	-	351	-	379	-

Table 3: Bovine manure culture results of dairy farms

	Positive		Negative		Total	
Fecal samples from beds	Count	Row (%)	Count	Row (%)	Count	Row (%)
Outdoors	3	4.8	60	95.2	63	100
Indoors	7	11.1	56	88.9	63	100
Total	10	-	116	-	126	-

Table 4: Culture results of raw milk different samples of dairy farms

	Positive		Negative		Total	
Milk samples	Count	Row (%)	Count	Row (%)	Count	Total (%)
Cows with clinical signs	7	58.3	5	41.7	12	100
Farm bulk milk tanks	5	7.3	64	92.7	69	100
Bulk milk transporters	0	0.0	67	100.0	67	100
Total	12	-	134	-	148	-

the feces will increase so it expands environmental contamination. There are also similar remarks reported by Hilton and Back (2003) and Whittington and Sergeant (2001). The difference between the positive and negative results of the groups was shown in Table 2.

The results of the bovine manure samples taken from the bedding of dairy farms are shown in Table 3. Of the samples taken from indoor and outdoor, only 7 and 3 were positive, respectively. When soil is contaminated with the *M. paratuberculosis* through the feces, there would be a reduction of 90-99% in apparent viable count of them. It is thought to be caused by binding of bacteria to soil particles which are excluded from culture by sedimentation process during the sample preparation. On the other hand, the organism is relatively susceptible to sunlight and drying and continuous contact with urine and feces reduces the longevity of the bacteria. Therefore, difference in bacterial count of manure in various conditions (e.g., shady and sunny conditions) in different periods is expected. Such a result might be due to the small number of samples.

Isolation of *M. paratuberculosis* from milk: Samples of bulk milk transporters were negative. Out of 63 samples taken from farm bulk milk tanks, 5 (7.3%) samples were positive. But the samples of cows showing the illness had 50% positivity (Table 4). Table 4 shows the results of milk samples from different sources and their discrepancies. It is obvious that the collected milk from multiple farms is mixed with each other in a milk collection center or bulk milk transporter. Therefore, the contaminated milk dilutes and subsequently chance of bacterial isolation decreases.

PCR and serological tests are less sensitive than fecal culture for detection of small number of bacteria particularly at initial stages of disease or diagnosis of subclinical forms of Johne's disease (Songer *et al*., 2005). For reduces the cost of bacterial culture test is suggested using pooled fecal culture for disease screening in herds which aren't contaminated or suspected with Johne's disease. This manner of fecal culture is carried out with mixture from feces of five to ten animals of the same age per pool. If a herd is infected, individual cultures or other tests would be necessary. Pooling samples reduces the number of fecal cultures necessary to determine infection, thereby reducing the cost of a large-scale Johne's disease control or eradication program. Although, the centrifugation of fecal samples might increase the number of fungal contamination in culture media, it increases the sensitivity of fecal culture test. In as much as immunomagnetic beads increase the sensitivity of *M. paratuberculosis* isolation rate from milk, they are recommended for isolation bacteria from farm bulk milk tanks and disease screening in dairy farms. The incidence is not regularly reported. However, according to the current report of bacteria isolation from different parts and economic importance of Johne's disease, it is logical that the prevalence rate of the disease in dairy farms is initially determined and afterwards the control programs are performed. It is essential to remark that the sale of calves and the transportation of animals and their manure are prohibited until the results of two consecutive fecal culture tests (with an intervals of six months) is not negative. Not only administration of educational workshops for operators of veterinary laboratories and veterinarians, but also making the owners of cattle herds aware of the importance of Johne's disease will be useful.

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