Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

# Comparison of Isolation Frequency of *Mycobacterium avium* subspecies *paratuberculosis* from Different Types of Samples

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ARTICLE HISTORY

Received: June 09, 2009
Revised: January 21, 2010
Accepted: February 24, 2010

**Key words:**
Comparison
Isolation
*Mycobacterium avium*
*Paratuberculosis*

**ABSTRACT**

Samples of feces, manure and milk of 137 dairy cattle herds were collected from different parts of Razavi Khorasan province of Iran. These herds were suspected of having Johne's disease. There were 505 faecal and manure samples and 148 raw milk samples prepared from cows affected by the clinical form of Johne's disease (n = 12), bulk milk-transporters (n = 67) and farm bulk milk tanks (n = 69). 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. Diagnostic test of *M. paratuberculosis* culture was positive in 29 out of 137 herds. Out of feces, manure and milk samples, 50 cases were found positive. The number of bacterial colonies varied from 1 to 250. The frequency of positive and negative fecal samples taken from cows with clinical signs (n=16) and without clinical signs of Johne's disease (n=363), 13(81.3%) and 15(4.1%) were positive, respectively. The difference in positive samples between two groups of manure samples taken from outdoors and indoors of farms was non significant. The difference in positive cases among three different types of milk samples was significant, but these differences between two groups of bulk milk-transporters and farm bulk milk tanks were non significant. Using Fisher exact test, the comparison between fecal and raw milk samples from cows with clinical signs showed non significant difference between two groups, but these differences based on the number of positive tubes and their colonies were significant.



**To cite this article:** Seyyedin M, T Zahraei and Najafi MF, 2010. Comparison of isolation frequency of *Mycobacterium avium* subspecies *paratuberculosis* from different types of samples. Pak Vet J, 30(3): 143-149.

## INTRODUCTION

Paratuberculosis, also known as Johne's disease, is a chronic granulomatous enteritis that affects domestic and wild ruminants. It is caused by *Mycobacterium paratuberculosis* (Grant *et al*., 1998). Because of its long incubation period, mainly subclinical or hidden forms of the disease are noticed. However, after a long subclinical phase, clinical signs such as cachexia and diarrhea can be recorded (Bhidea *et al*., 2006). The caustive agent of paratuberculosis is a gram-positive, non-motile, non-spore-forming, acid-fast and facultative anaerobic intracellular bacterium. However, some non-acid fast, lightly acid-fast and cell wall-deficient types are also encountered. The bacilii generally occurs in clumps linked together by a network of intercellular filaments (Ayele *et al*., 2001; Henderson *et al*., 2003). It grows slowly and is a non-chromogenic, biochemically unreactive organism that requires mycobactin-enriched media for growth. *M. paratuberculosis* contains approximately 15-20 copies of the insertion sequence IS900 (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 colour (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).

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. 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 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 slightly more sensitive than conventional culture systems but is more expensive and requires specific instruments (Songer and Post, 2005; Gumber and Whittington, 2007; Radostits *et al*., 2007).

The main aim of this study was comparison of isolation frequency of *Mycobacterium avium subspecies paratuberculosis* from different types of samples and the detection of subclinical forms of Johne's disease and recognition of infected dairy herds.

## MATERIALS AND METHODS

### Sampling

From May 2006 to October 2007, 505 feces and manure samples and 148 raw milk samples including samples from the bulk milk transporters, from cows with clinical signs of paratuberculosis, and from bulk milk tanks were taken from 137 dairy farms from different parts in Razavi Khorasan province, Iran. The samples were randomly collected from herds suspected of having Johne's disease. With the exception of samples from 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 from where fecal samples were also taken. No manure sample was taken from farms from which milk or fecal samples were collected. Also, none of the farms was bacteriological tested for detection of *M. Paratuberculosis* before the study.

### Medium preparation

For cultivation of *M. Paratuberculosis,* Herrold's medium was prepared according to OIE (2004). In order to prevent bacterial and fungal contamination, 50 mg/L nalidixic acid, 50 mg/L amphotericin B and 50 mg/L vancomycin were added to the medium.

### Fecal samples

A total of 379 fecal samples were collected from dairy farms. The samples were directly taken from the cows' rectum. Among these, 16 samples were taken from cows with advanced clinical signs of Johne's disease, 53 were taken from cows without clinical signs but having contact with clinical cases of Johne's disease and the rest of the samples were from animals that had no contact with clinical cases of paratuberculosis. Following collection, samples were immediately placed in a container with ice packs and transported to the laboratory, where 10g of each sample was stored at -70ºC until use for bacterial culture.

### Manure samples

A total of 126 manure samples were taken from different herds with the history of Johne's disease. Among these, 63 were sampled from outdoor dairy herds, and the other samples were taken under similar conditions from the same herds indoors. After collection, all samples were transported to the laboratory under cold condition and stored as stated above.

### Raw milk samples

A total of 148 raw samples were obtained from different regions. Out of these, 67 samples were from bulk milk transporters with the capacity of 10000-15000 liters, 69 samples were from the farm bulk milk tanks having the history of Johne's disease, while 12 samples were from cows showing clinical signs of Johne's disease (fecal samples were taken simultaneously from these cows). After collection, all samples were transported to the laboratory under cold condition. Appropriate amount of milk sample (50 ml) was centrifuged at 4ºC (3100 g, 15 min). Cream and pellet fractions were mixed together and kept at -70ºC for bacterial culture.

### Culture of fecal and manure samples

Fecal and manure samples were cultured on Herrold's egg yolk agar medium (HEYM) with and without mycobactin J (OIE 2004). 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.

### Milk culture

The isolation was done according to Dundee *et al*. (2001), Grant *et al*. (2005) and Gao *et al*. (2005), with some modifications. Briefly, each pellet and cream fraction was re-suspended in 20 ml of Hexadecylpyridinium chloride (HPC) 0.75% (w/v) and incubated for 5h at room temperature. Following incubation, samples were centrifuged at 4ºC (Bhidea *et al*., 2006) for 15 min, 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 Herrold's media, similar to fecal culture.

### Colonial morphology

The isolated bacteria from feces, manure and milk were examined for colony morphology as well as acid fast staining.

### DNA extraction

DNA extraction was performed by taking single colonies. The single bacterial colony was taken from the HEYM medium containing mycobactin J and resuspended in 50 µl distilled water in a screw-cap micro-centrifuge tube. The samples were boiled for 20 min prior to being centrifuged at 14000g for 5 min to settle cell debris. Then 2 µL of supernatant containing the genomic DNA, was used for Nested PCR-IS900 amplification (Hosek *et al*., 2006).

### PCR

PCR assay was performed for confirmation of *M. paratuberculosis* and was done according to 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 MgCl2, 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 Mastercycler 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'). The second PCR products were analyzed on a 1.2% agarose gel containing 0.5% ethidium bromide and visualized by UV-light transilluminator.

**Statistical analyses**

Frequency of positive and negative samples of different types was computed. In order to see the magnitude of variation, the data were analyzed statistically using Pearson Chi square test and Fisher exact test.

## RESULTS

In this study, we identified and confirmed that cattle were susceptible to Johne's disease on the dairy cattle herds in Razavi Khorasan province, Iran. Also, application of different types of samples for detection of paratuberculosis was evaluated. Diagnostic test of *M. paratuberculosis* culture was positive in milk, manure and fecal samples of 29 out of 137 herds (21.17%). Out of 653 samples, 50 cases (7.66%) were found positive for *M. paratuberculosis* after cultivation.

**Colonial morphology**

It was possible to observe *M. paratuberculosis* colonies on Herrold's media containing mycobactin J from the 9$^{th}$ week onward. In the beginning, colonies were small, about 1 mm in diameter, but their diameter gradually increased up to 3 mm. The colonies were spherical, semi-transparent or translucent, bright in appearance, smooth to somewhat rough and off-white to yellow in colour (Fig. 1). The number of bacterial colonies varied from 1 to 250. The highest and the lowest number of colonies were found in the fecal samples of clinically Johne's-affected cows and raw milk samples of farm bulk milk tanks, respectively (Table 1). All the isolated bacteria were confirmed through colony morphology, acid fast staining and Nested PCR–IS900 assay.

**Nested PCR**

The all isolated strains were PCR positive and target gene with 210 bp sizes was observed. This corresponds with size of band of positive control (*M. avium* subsp. *paratuberculosis* strain 316F) (Fig. 2).

**Isolation of *M. Paratuberculosis* from fecal and manure samples**

Table 2 shows the frequency of the positive and negative fecal samples from cows with and without clinical signs of Johne's disease. Among 16 samples taken from the cows with clinical signs, 13(81.3%) were positive after cultivation, while this number for the 363 cows without clinical signs of Johne's disease was 15(4.1%). This indicates that there are considerable differences in both groups. Further analysis by Pearson Chi square test revealed that the difference between the two groups was statistically significant ($p<001$). According to the results, the number of positive tubes and number of colonies were closely related.

The cows without clinical signs belonged to two groups either exposed to or non-exposed to the clinical cases of Johne's disease. As shown in Table 3, out of 53 samples from animals exposed to sick cows, 11(20.8%) were positive, while this value for non exposed group was 1.3% (4 samples from 310 samples). The difference between the two groups was statistically significant ($P<0.05$).

The results of the manure samples taken from dairy farms are shown in Table 4. Out of 63 samples taken from outdoors and 63 samples from indoors, only 3(4.8%) and 7(11.1%) were positive, respectively. The difference between these two groups was non significant.

**Isolation of *M. paratuberculosis* from milk**

None of the samples taken from bulk milk transporters was found positive for M. *paratuberculosis*. Out of 69 samples taken from farm bulk milk tanks, 5(7.3%) were positive. But the samples from cows showing the illness signs showed 58.3% positive (Table 5). The differences in the frequency of positive cases among three groups were significant ($P<0.05$). Since the number of positives in the samples related to bulk milk transporters and farm bulk milk tanks were not different, positives samples from the milk of cows showing signs were removed from the analysis and then other two groups were compared with Fisher test which revealed non significant difference between the two groups (Table 6).

**Comparison between fecal and raw milk samples from cows with clinical signs**

Using the exact Fisher test for comparing the results of both groups, it was observed that there were no significant differences between samples from fecal and raw milk of clinical cases (Table 7), but there were some variation between the number of positive tubes and the colonies in both groups. So the numbers of colonies were grouped in three ranges, 11-50, 51-100, 101-250, and the total positive tubes related for each group were recorded. Since probability value was smaller than 5% ($P = 0.000$), the difference between the two groups was statistically significant (Table 8).

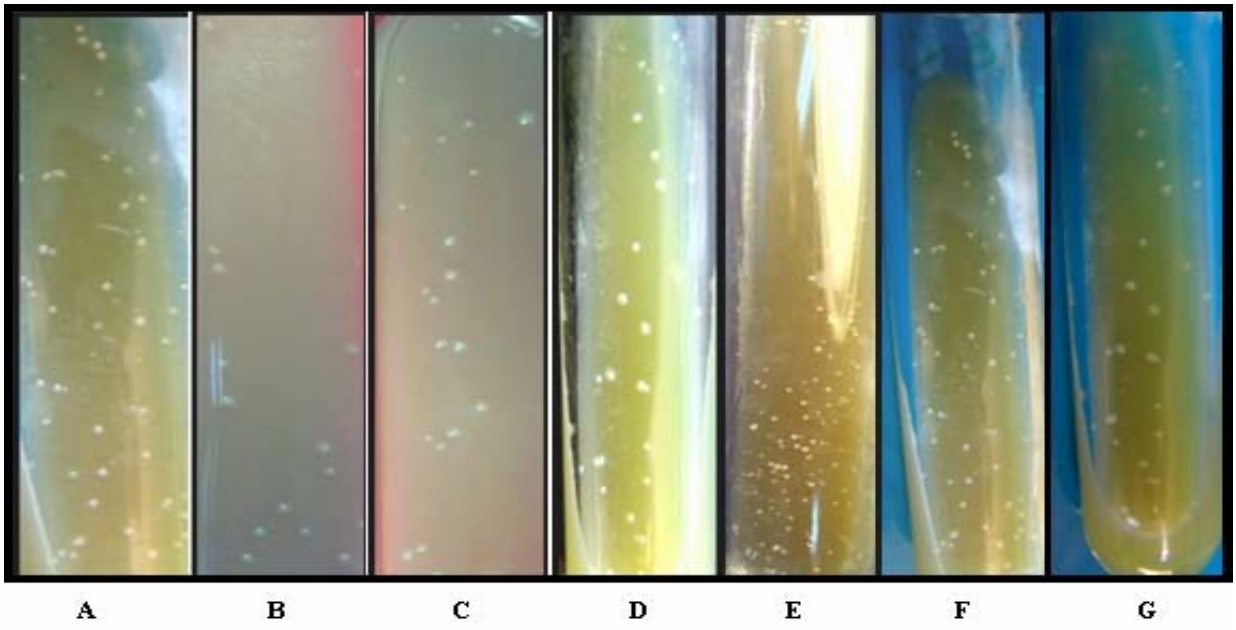


**Fig. 1:** ***Mycobacterium avium subsp paratuberculosis*** **colonies on Herrold's egg yolk medium containing mycobactin J. E = colonies isolated from cows showing clinical signs of Johne's disease. A, B and C = colonies isolated from raw milk samples and D, F, and G = colonies isolated from fecal samples of the cows without the sings of Johne's disease.**

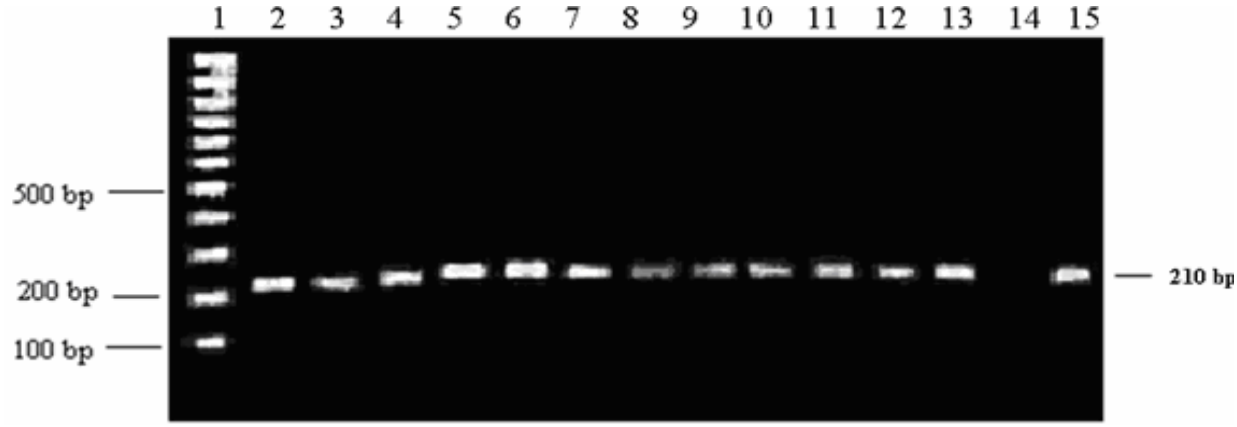


**Fig. 2: The result of Nested-PCR of** ***Mycobacterium avium subsp. paratuberculosis*** **isolated from feces, manure and milk samples growth in specific media. Lane 1 molecular weight of standard (100 bp), Lane 2-13 isolated strains, Lane 14 negative control (sterile buffer), Lane 15 positive control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

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

| Colony count | Type of samples | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Raw milk | | | Feces | | | Manure | |
| | Cows with clinical signs | Farm bulk milk tanks | Bulk milk transporters | Cows with clinical signs | Cows without clinical signs (expose) | Cows without clinical signs (Non-expose) | 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 |

**Table 2: The frequency of positive and negative fecal samples for cows with and without clinical signs of Johne's disease**

| Fecal samples | Positive | | | Negative | | | Total | |
|---|---|---|---|---|---|---|---|---|
| | Count | Row percent | Column percent | Count | Row percent | Column percent | Count | Total percent |
| With clinical signs | 13 | 81.3 | 46.4 | 3 | 18.7 | 0.9 | 16 | 100.0 |
| Without clinical signs | 15 | 4.1 | 53.6 | 348 | 95.9 | 99.1 | 363 | 100.0 |
| Total | 28 | - | 100.0 | 351 | - | 100.0 | 379 | - |

Pearson Chi-square: 133.20, df = 1, P = 0.000

**Table 3: The frequency of positive and negative fecal samples for cows without clinical signs either exposed to or non exposed to the clinical cases of Johne's disease**

| Fecal samples (without clinical signs) | Positive | | | Negative | | | Total | |
|---|---|---|---|---|---|---|---|---|
| | Count | Row percent | Column percent | Count | Row percent | Column percent | Count | Row percent |
| Exposed to | 11 | 20.8 | 73.3 | 42 | 79.2 | 12.1 | 53 | 100 |
| Non-exposed | 4 | 1.3 | 26.7 | 306 | 98.7 | 87.9 | 310 | 100 |
| Total | 15 | - | 100 | 348 | - | 100 | 363 | - |

Pearson Chi-square: 43.29, df = 1, P- value = 0.000

**Table 4: The frequency of positive and negative manure samples**

| Manure samples | Positive | | | Negative | | | Total | |
|---|---|---|---|---|---|---|---|---|
| | Count | Row percent | Column percent | Count | Row percent | Column percent | Count | Row percent |
| Outdoors | 3 | 4.8 | 30 | 60 | 95.2 | 51.7 | 63 | 100 |
| Indoors | 7 | 11.1 | 70 | 56 | 88.9 | 48.3 | 63 | 100 |
| Total | 10 | - | 100 | 116 | - | 100 | 126 | - |

Pearson Chi-square: 1.74, df = 1, P = 0.19

**Table 5: The frequency of the positive and negative raw milk samples taken from bulk milk transporters, farm bulk milk tanks of dairy farms and the milk samples of the cows showing clinical signs of Johne's disease**

| Raw milk samples | Positive | | | Negative | | | Total | |
|---|---|---|---|---|---|---|---|---|
| | Count | Row percent | Column percent | Count | Row percent | Column percent | Count | Row percent |
| Bulk milk transporters | 0 | 0 | 0 | 65 | 100 | 48.5 | 65 | 100 |
| Farm bulk milk tanks | 5 | 7.3 | 41.7 | 64 | 92.7 | 47.8 | 69 | 100 |
| Cows with clinical signs | 7 | 58.3 | 58.3 | 5 | 41.7 | 3.7 | 12 | 100 |
| Total | 12 | - | 100 | 134 | - | 100 | 146 | - |

Pearson Chi-square:45.86, df = 2, p = 0.00

**Table 6: The frequency of the positive and negative raw milk samples taken from the bulk milk transporters and farm bulk milk tanks of dairy farms**

| Raw milk samples | Positive | | | Negative | | | Total | |
|---|---|---|---|---|---|---|---|---|
| | Count | Row Percent | Column Percent | Count | Row Percent | Column Percent | Count | Row Percent |
| Bulk milk transporters | 0 | 0 | 0 | 65 | 100 | 50.4 | 65 | 100 |
| Farm bulk milk tanks | 5 | 7.3 | 100 | 64 | 92.7 | 49.6 | 69 | 100 |
| Total | 5 | - | 100 | 129 | - | 100 | 134 | - |

Fisher exact test: p = 0.06

**Table 7: The frequency of the positive and negative fecal and raw milk samples taken from cows showing clinical signs of Johne's disease**

| Samples | Positive | | | Negative | | | Total | |
|---|---|---|---|---|---|---|---|---|
| | Count | Row percent | Column percent | Count | Row percent | Column percent | Count | Row percent |
| Feces | 13 | 81.3 | 65 | 3 | 18.7 | 37.5 | 16 | 100 |
| Raw milk | 7 | 58.3 | 35 | 5 | 41.7 | 62.5 | 12 | 100 |
| Total | 20 | - | 100 | 8 | - | 100 | 28 | - |

Fisher exact test: P = 0.231

**Table 8: Comparison of the number of positive tubes and their colonies of fecal and milk samples of clinical cases of Johne's disease**

| Colony count | Milk samples | Fecal samples |
|---|---|---|
| 11-50 | 10 | 0 |
| 51-100 | 1 | 21 |
| 101-250 | 0 | 12 |
| Total | 11 | 33 |

Pearson Chi-square: 34.9744, df = 2, p = 0.000

## DISCUSSION

In this study, we identified and confirmed that the cattle were susceptible to Johne's disease on the dairy cattle herds in Razavi Khorasan province, Iran. Razavi Khorasan province is one of the largest provinces in Iran in terms of size and animal populations. It is located in the north east of Iran and borders with Afghanistan and Turkmenistan.

As explained in Tables 2 and 3, significant differences were observed in the frequency of positive animals between the cows with and without clinical signs. Also, differences were seen in the number of positive tubes and the number of organism colonies (Table 1). The transmission of *M. paratuberculosis* via milk and colostrum increases the risk of paratuberculosis infection in the herd (Manning *et al*., 1988). Also, with the continous development and phase-switching of disease from subclinical to clinical, bacterial shedding rate in milk and especially in the feces increases. This increase in bacterial shedding, specifically in feces, causes extension of environmental contamination and increases the frequency of affected animals. Similar findings have been reported by Whittington and Sergeant (2001). When the feces containing the *M. paratuberculosis* mix with soil, there is a reduction of 90-99% in the apparent viable count of the organism. This is probably caused by binding of bacteria to soil particles, which are excluded from culture by sedimentation during sample preparation (Whittington *et al*., 2003). Also, attachment to soil occurs with other non tuberculous mycobacteria (Brooks *et al*., 1984). *M. Paratuberculosis* is relatively susceptible to sunlight and drying, and continuous contact with urine and feces reduces the longevity of the bacteria (Jørgensen, 1977).

It was expected that there would be a significant difference between the manure samples collected from indoors and outdoors, but the differences were not significant statistically (Table 4). This is probably related to the small number of samples and their conditions, including conditions of collection, storing and period of storing manure at various farms. *M. paratuberculosis* may be transmitted prenatally or postnatally, when most infection occurs through the fecal-oral route (Sweeney, 1996). The commonest route of infection with *M. paratuberculosis* is through nursing from an infected dam (via contaminated teats or direct shedding of the organism into the colostrum/milk) or ingestion of contaminated feed and water. Cows affected by clinical or subclinical form of Johne's disease can shed a large number of *M. paratuberculosis* into colostrum or milk. Thus, colostrum or milk of infected cows, if fed to calves, could serve as a potential source of infection (Radostits *et al*., 2007). Removal of the calf from the dam at birth before nursing is one strategy that helps reduce the incidence of Johne's disease (Rossiter and Burhans, 1996).

There was significance difference between the milk samples belonging to clinical cases and the other two groups. Therefore, a comparison was done between the milk samples taken from bulk milk transporters and farm bulk milk tanks. However, no significant difference was observed statistically between both groups, which seems to be due to the small number of samples. Nearly 10% of subclinically infected cows and 50% of animals showing clinical paratuberculosis excreted the agent in the milk (Pavlas, 2005). Also, according to some of reports do not different between diagnostic sensitivity *M. paratuberculosis* from milk and feces under identical conditions (Sweeney *et al*., 1992; Pillai and Jayarao, 2002), but on the other hand, the number of *M. paratuberculosis* shed through milk compared with feces is less (Radostits *et al*., 2007). For this reason a comparison was made between the frequency of isolation of bacteria from milk and the feces of cows showing clinical signs of Johne's disease. There were numerical differences between the two types of samples, but the differences were not significant statistically. This lack of statistical difference may be due to the small number of samples. Therefore, comparison was done between the number of the positive tubes and their bacterial colonies. In this comparison, statistically significant differences were seen between isolation of bacteria from milk and feces.

It is essential to remark that the sale of calves and the transportation of animals and their manure may be prohibited until the results of two consecutive fecal culture tests (with an interval of six or 12 months) are negative. Not only organization of educational workshops for scientists of veterinary laboratories and veterinarians, but also making the owners of cattle herds aware of the importance of Johne's disease will be useful. In addition to fecal samples, examination of manure samples and milk samples taken from farm bulk milk tanks can help to detect subclinical forms of Johne's disease. In conclusion**,** according to the current report of bacterial isolation from different places and the economic importance of Johne's disease, it is logical and essential that the prevalence rate of the disease in dairy cattle herds is initially determined by at least two different types of samples and tests and afterwards the control programs are adopted.

**Acknowledgments**

We would like to acknowledge the collaboration of the management and personnel of the Department of Microbiology & Immunology, College of Veterinary Medicine, Tehran and the collaboration of the management and staves of biotechnology research department of Razi Vaccine & Serum Research Institute of Mashhad. Special thanks go to the management and staves of veterinary headquarters in Razavi Khorasan province, particularly Dr. Javad Alami who collaborated with us for sending the samples.

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