Detection of *Mycobacterium avium subsp. paratuberculosis* in manure and milk filters of apparently healthy dairy herds in Hesse, Germany

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Abstract: Aim and Background: Examination of environmental samples such as manure and soil could be considered as an alternative screening technique for the detection of MAP. So it may improve the efficiency of Johne's Disease control strategies especially in areas with low MAP prevalence. This study was conducted to evaluate and investigate the usefulness of environmental samples and especially milk-filter samples in the improvement of MAP diagnosis. In addition, the present work aims to investigate the possibility of using these samples as an alternative cheaper diagnostic matrix for MAP- detection in dairy herds. Methodology: Manure and milk filter samples were collected from 63 German dairy cattle herds in Hessen to be investigated for the presence of ***Mycobacterium avium* subsp. *paratuberculosis*** (MAP). All herds included were tested for MAP status in a previous study by screening with the Pourquier® ELISA and bacterial isolation from individual faeces and/or milk samples. Results: MAP was identified in 11.2% and 3.6% of tested manure and milk filter samples respectively. Grown colonies were characterized by phenotypic characters, Ziel Neelsen stain and by PCR amplification of MAP the IS*900* sequence and the f*57* gene. Conclusion: The current study is the first report of the isolation of MAP from milk filters highlighting the usefulness of this kind of sampling in the identification of MAP on herd level. Moreover, such environmental sampling could aid in the future as a cheaper alternative screening techniques for detecting MAP status on herd level.
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Abbreviations:
JD Johne's disease
MAP *Mycobacterium avium* subsp. *paratuberculosis*

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiological agent of Johne's disease (JD) and causes chronic granulomatous enteritis in cattle. An important factor in the persistence of JD in dairy farms, is that clinically as well as sub-clinically affected cattle contaminate the surrounding environment with huge number of the bacteria through their faeces and subsequently act as a source of infection for other animals [1-3].

According to previous studies, examination of environmental elements such as manure, soil and pastures could be considered as an alternative screening technique for the detection of MAP leading to the improvement of the efficiency of JD control strategies [3-5]. Most control programs depended mainly on the serological examination of milk and/or blood samples using tests like the commercial Enzyme-Linked Immunosorbent Assays (ELISA). Other programs that depend on bacteriological examination of individual animals, and/or pooled fecal samples accompanied with ELISA tests are widely accepted to support the disease control on both national and international levels [1]. Although it was previously recommended to test different environmental samples from the surrounding of examined herds, studies on environmental sampling have not included any milk filter samples [3-5]. The Purpose of the present study is to evaluate an easy to collect matrix for detecting MAP in cattle herds that ensure a correct diagnostic value. The motivation was to investigate the usefulness of environmental samples and especially milk-filter samples as an alternative cheaper diagnostic matrix for MAP-detection in dairy herds.

2.Material and methods

Dairy Herds, Milk filters and Manure samples

In the present study 63 apparently healthy dairy herds in Hessen (40 to 100 Holstein lactating cows / herd, with total number of 4515 lactating cows) were included. These dairy herds were previously tested for MAP status in a previous study by screening with the specific Pourquier® ELISA and bacterial isolation from individual faeces and/or milk samples [6].

Milk filter samples (approximately 30 x 5 cm) and manure samples (400-500 g/sample) were collected from all these 63 herds. Manure samples were collected from areas with high cow traffic. Totally four samples per herd were taken from 4 different locations of the stable, pooled and treated as a single sample representing this farm.

Isolation of MAP from milk filters

Sixty three milk filter samples were investigated for the presence of MAP after modifications made to a previous method [7]. Milk filters (7 cm) were cut vertically in small strips and suspended in 30 ml fresh 0.75% (HPC) Hexadecyl-Pyridinium-Chloride (Sigma, Germany) in a 50 ml plastic tube and shaken at 200 rpm for 30 min. Then the samples were kept for 18-20 hrs at room temperature. After removing the strips with a sterile forceps the suspension was centrifuged at 2500x *g* for 15 min. Approximately 25 ml of the supernatant was decanted. Finally, the pellet was divided over three slants of Herrold's Egg Yolk Medium (HEYM-MJ) containing Mycobactin J and ANV (Becton Dickinson GmbH, Heidelberg, Germany).

Isolation of MAP from Manure samples

Sixty three manure samples were collected. These samples were investigated for the presence of MAP after modifications made to a previous protocol [7]. Totally four manure samples per herd were taken from 4 different locations of the stable, pooled and treated as a single sample representing this farm. Each sample was first homogenized. Three grams were taken and suspended in 30 ml fresh 0.75% HPC in a 50 ml plastic tube and shacked at 200 rpm for 30 min. The mixture was then kept for 1-2 min at room temperature. After that the supernatant was carefully transferred in a new tube. The sample was kept for 18-20 hrs at room temperature followed by centrifugation at 2500x *g* for 15 min. The supernatant was then carefully decanted. The pellet was carefully re-suspended and inoculated in three tubes of HEYM-MJ.

Identification of isolates

Phenotypic characters

All tubes were incubated at 37°C and were monitored biweekly at weeks 6, 8, 10, 12, 16 and 20 for the detection of small white to yellow half ball shape or pinhead Map colonies. Single colonies from visible growth in all tubes were stained with Ziehl-Neelsen stain (ZN) according to the manufacturer's instruction (Merck, Darmstadt, Germany) before being subcultured on HEYM agar [6].

Molecular identification

All obtained isolates were confirmed as Map after the application of 2 Map specific PCR assays targeting the IS900 and f57 DNA fragments using PCR conditions that were described previously [8,9]. The primer set used in the IS900 PCR was the primer pair **TJ1** GCT GAT CGC CTT GCT CAT **TJ2** CGG GAG TTT GGT AGC CAG TA. While the primers used in the PCR amplification of the F57 gene were **F 57** CCT GTC TAA TTC GAT CAC GGA CTA GA and **R 57** TCA GCT ATT GGT GTA CCG AAT GT. PCR was performed in a final volume of 50 µl containing 5µl from the isolated DNA (obtained by direct bacterial boiling for 10 min), 1.25 U *Taq* polymerase, 1x PCR buffer containing 1.5 mM $MgCl_2$, 200 µM dNTP and 2 µM of each primer. The thermal cycler parameters used for IS900 amplification included an initial 95ºC incubation step for 10 minutes followed by 30 cycles of denaturation at 95ºC for 1 min., annealing at 58ºC for 1 min. and extension at 72ºC for 2 min. with a final extension step at 72ºC for 10 min.

The thermocycler amplification parameters for the F57 gene were 1 cycle of denaturation at 94°C for 4 min. followed by 40 cycles of denaturation at 94°C for 45 sec., annealing at 68°C for 45 sec. and extension at 72°C for 45 sec. followed by a final extension step at 72°C for 10 min.

3.Results and discussion.

The identification of a MAP infected dairy herd is a difficult task. The use of the environmental materials in addition to the traditional samples to detect hidden foci of the infection can save time, money and effort. This can only be achieved if the use of the environmental materials has the same diagnostic value as the traditional ones.

Cultural manure and milk-filter-sample results in relation to the ELISA-results are given in **Table 1**. In examined dairy herds, where no positive ELISA reactors (11 herds), there were two positive cultural results one manure sample and one milk filter sample. In herds, with only one positive ELISA reactor (38 herds), there were 5 positive cultural results 4 manure samples and one milk filter sample. While herds which had 2 or 3 positive ELISA reactors (8 herds), all manure samples and milk filter samples were negative for isolation of MAP. Two positive manure samples were obtained from herds which had 5 positive ELISA reactors.

Seven out of 63 manure samples yielded positive cultural results. Because of a fully contamination with fungi and different *Bacillus* species only 55 of 63 milk filter samples could be evaluated. The cultural examination revealed only two positive results as shown in **Table 2** and **Figure 1**.

The species identification according to the typical culture morphology of suspected colonies could be confirmed additionally by MAP specific PCRs (IS*900,* f*57*) in all cases **Figure 2**. Nearly one half of all positive manure samples were detected in herds with no detectable MAP- shedders. Two positive samples could be observed in herds with only one shedder, while the other two positive samples were found in herds with two and four MAP-shedding cows each. With regard to the two positive milk filters, only one of them originated from a herd with a single MAP- shedding cow (**Table 2**).

The low detection level of MAP in these kind of samples compared to the results of ELISA testing might be referred to different possible reasons: In these investigated herds clinical cases of Johne's disease had never been reported or are not known in the past. Moreover, it is well known that the number of excreted MAP by subclinically infected animals is only low 1 to 10^2/g faeces [10,11]. In consequence, the number of bacteria in the environmental surrounding (cow alleyways, manure, slurry etc.) should also be low. For reasons of practicability, Samples were collected from 4 locations in most frequently used cow alleyways. This comparatively low number of sampling points could be a reason for the observed low cultural detection rate of MAP in manure seen in the present study.

False positive ELISA reactors could also be conceivable for this discrepancy. They were also reported [12-14] This may be induced by the exposure of the tested animals to other mycobacterial subspecies than MAP. The ELISA test used for screening of these herds has a specificity of nearly 99%. The majority of the ELISA positive herds had only one ELISA-positive cow. In contrast ELISA negative test results are consequences of the comparatively low sensitivity of this diagnostic tool at animal level. The ELISA test used has a comparable low sensitivity of 54.5% [15,16]. Consequently, nearly every second MAP- infected cow will not be detected by this ELISA test. Additionally, the fecal culture as it is commonly known has an even lower sensitivity because MAP is excreted periodically. This would explain the two MAP-positive environmental samples in one farm without any serologically positive and/or MAP-shedding cow [17,18]. On the other hand the previous study did not examine every cow in these herds, so it is conceivable that MAP shedders were not found [6]. According to the presented results, Only two positive culture of the 55 milk filter samples which could be evaluated (3.6%), this results is less than obtained by Cashman *et al.* [19] which found 20% positive Milk filter samples in the examined 59 Irish dairy herds cattle. The detection rate of MAP in milk filters was much lower than in manure samples. This might be caused by methodical problems as the applied cultural technique is actually determined only for fecal material. As feces and manure being the first source of MAP furthermore hygienic handling within the dairies should prevent the secondary contamination of milk and equipment with these materials.

All herds with positive milk filter samples were also positive in manure samples. Providing a contamination after sample collection this seems to be a clear evidence for a secondary contamination of milk with MAP. Further studies should prove this for example by comparing MAP detections in milk filters with the number of coliform germs as a hygienic indicator in these samples.

Table 1. Relationship between Pourquier®- ELISA test and cultural results of manure and milk filter samples

Number of positive ELISA reactors in dairy herds	Total number of dairy herds	Positive cultural results	
		Manure samples	Milk filter samples
0	11	1	1
1	38	4	1
2	6	0	0
3	2	0	0
4	0	0	0
5	6	2	0
Total number	63	7	2

Table 2. Relationship between results of cultural tests of individual fecal samples and cultural results of manure, milk filter samples

Number of positive fecal samples in dairy herds	Total number of dairy herds	Positive cultural results	
		Manure samples	Milk filter samples
0	51	3	1
1	7	2	1
2	4	1	0
4	1	1	0
Total number	63	7	2

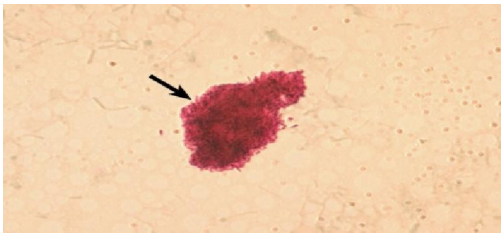

Fig 1. Acid fast bacilli under oil immersion lens.

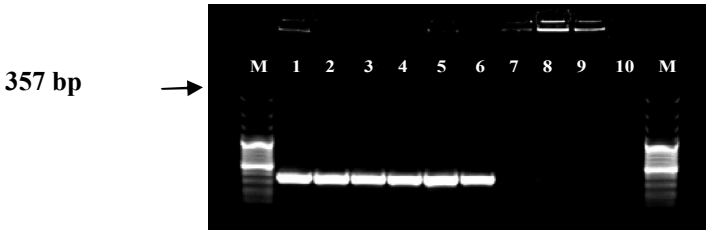

Fig 2. Molecular identification of *M. avium* subsp. *paratuberculosis*.
IS*900* PCR (357 bp). M: 100 bp DNA ladder, **lane 1:** Colonies retrieved from milk filter sample (Hes12), **lane 2:** Colonies retrieved from milk filter sample (Hes57), **lane 3:** Colonies retrieved from manure sample (Hes12), **lane 4:** Colonies retrieved from manure sample (Hes33) **lane 5:** Coded IS900 positive sample from our DNA bank, **lane 6:** positive control (ATCC® BAA-968 type strain), **lane 7:** *M. avium* subsp. *avium* (ATCC 44156), **lane 8:** *M. phlei* (ATCC 43239), **lane 9:** *M. scrofulaceum* (ATCC 43992), **lane 10:** negative control.

4. Conclusion:

The current study highlights the usefulness of using two sample to collect matrices like manure and milk-filters to simplify the diagnostic method of MAP on herd level. Moreover, such environmental sampling could aid in the future as a cheaper alternative screening technique in regional control programs to detect infected herds. Future studies on environmental sampling will certainly increase the overall sensitivity of these methods.

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