

Detection of *Mycobacterium avium* subspecies *paratuberculosis* in Swiss dairy cattle by culture and serology

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Summary

Faecal samples from 186 dairy cows representing ten commercial dairy herds with sporadic clinical paratuberculosis (group A), and from 100 dairy cows from herds without a history of paratuberculosis (group B) were cultured for *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Two different decontamination methods, a NaOH/oxalic acid method and treatment with 0.75% hexadecylpyridinium chloride (HPC) were performed prior to inoculation of Loewenstein-Jensen agar slants with and without mycobactin. Cultures were incubated for 16 weeks. Acid-fast staining bacteria were identified as MAP on the basis of mycobactin dependency and by PCR-RFLP analysis of the IS1311-insertion element of *M. avium*. MAP was grown from 15 out of 186 group A animals (8.1%) whereas faecal culture for MAP was consistently negative in group B. The growth rate of MAP was significantly higher (8.1% vs. 1.6%) and the contamination rate of cultures was significantly lower (17.6% vs. 21.5%) in faecal samples decontaminated with NaOH/oxalic acid than with HPC-treated faecal samples ($p < 0.01$, McNemar's test). Atypical mycobacteria which were grown from 46.8% of NaOH/oxalic acid treated specimens were not obtained from any of the HPC-treated samples. A commercial ELISA with MAP-lipoarabinomannan as the antigen was used to detect MAP-antibodies in unabsorbed sera from all animals. The percentage of ELISA-positive cows was 16.8%. Overall agreement between antibody detection and MAP-positive faecal culture was 15.4%.

Key words: *Mycobacterium avium* subspecies *paratuberculosis*, cattle, diagnosis, culture, serology

Kultureller und serologischer Nachweis von *Mycobacterium avium* subspecies *paratuberculosis* bei Schweizerischen Milchkühen

Kotproben von 186 Kühen aus zehn Beständen mit sporadischer klinisch manifester Paratuberkulose (Gruppe A) und Kotproben von 100 Kühen aus Beständen ohne anamnestic Hinweise auf Paratuberkulose wurden kulturell auf *Mycobacterium avium* subspecies *paratuberculosis* (MAP) untersucht. Die Kotproben wurden parallel mit NaOH/Oxalsäure und mit 0.75% Hexadecylpyridiniumchlorid (HPC) dekontaminiert und auf Loewenstein-Jensen Schrägagar mit und ohne Mykobaktin verimpft. Die Kulturen wurden 16 Wochen bebrütet und auf das Wachstum von säurefesten Bakterien untersucht. MAP wurde anhand der Mykobaktin-abhängigkeit und durch PCR-RFLP Analyse des IS1311-Insertionselementes identifiziert. MAP wurde aus dem Kot von 15 Tieren der Gruppe A (8.1%) angezüchtet, war hingegen im Kot der Tiere der Gruppe B kulturell nicht nachweisbar. In NaOH/Oxalsäure-dekontaminierten Kotproben war die Nachweisrate von MAP signifikant höher (8.1% vs. 1.6%) und der Anteil kontaminierter Kulturen war signifikant geringer (17.6% vs. 21.5%) als in HPC-dekontaminierten Kotproben ($p < 0.01$, McNemar's test). Atypische Mykobakterien waren in HPC-dekontaminierten Kotproben kulturell nicht nachweisbar, wurden hingegen aus 46.8% der NaOH/Oxalsäure-behandelten Kotproben isoliert. In einem kommerziellen ELISA mit MAP-Lipoarabinomannan als Antigen waren bei 16,8% der 286 Kühe Antikörper gegen MAP nachweisbar. Dabei erbrachten das kulturelle und serologische Nachweisverfahren in 15,4% der Fälle übereinstimmend positive Resultate.

Schlüsselwörter: *Mycobacterium avium* subspecies *paratuberculosis*, Rind, Nachweis, Kultur, Serologie

Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of paratuberculosis (John's disease), a granulomatous incurable enteropathy affecting primarily wild and domestic ruminants. The disease has been established as an increasingly important cause of morbidity and significant economic loss in the dairy and beef cattle industry (Merkal et al., 1975; Buergelt and Duncan, 1978; Jones, 1989; Nordlund et al., 1996). MAP-infected cattle may also have public health implications since the agent has been linked to a human chronic granulomatous ileitis (Crohn's disease; Bull et al., 2003). In Switzerland, data about the prevalence as well as the clinical and economic impact of bovine paratuberculosis are scarce. A herd-level seroprevalence of 8.0% was determined in a serosurvey at nationwide level nearly one decade ago (Stärk et al., 1997). Currently, the disease is diagnosed only rarely, on average so far with six outbreaks per year (Swiss Federal Veterinary Office, 2003). However, a recent survey (2002) of 1384 bulk milk samples from different regions in Switzerland using the PCR amplification of the MAP-specific IS900 DNA sequence revealed 19.7% positive samples, thus indicating a rather high occurrence of subclinical MAP-infections in the Swiss dairy stock (Corti and Stephan, 2002). Considering the pathogenic significance in cattle as well as the public health implications due to probable involvement of MAP in Crohn's disease, efforts to analyse the prevalence of MAP in the Swiss cattle population to assist in deciding whether a control program is necessary seem reasonable. The present study aims at culturing MAP from bovine faeces by using two decontamination protocols. Moreover, our report outlines the application of an ELISA to examine bovine blood serum for MAP-antibodies.

Animals, Materials and Methods

Animals

Two groups of animals were enrolled for the study. Group A consisted of 186 dairy cows aged from 2 to 15 years, representing 10 commercial dairy herds which were chosen on a history of sporadically clinical paratuberculosis registered by the FVO. Herd sizes ranged from 5 to 35 animals. In these herds, single clinical cases of paratuberculosis have been diagnosed in the run-up to the present study. Group B included 100 dairy cows selected amongst animal patients admitted to the Clinic of Veterinary Internal Medicine, University of Zurich, Switzerland, for reasons other than intestinal diseases. These animals, each of which represented a single dairy herd without a past medical history of clinical paratuberculosis, ranged in age from 2 to 11 years. Samples were collected from

April until August 2002. Faeces were collected from the rectum and were processed for culture within 24 h after collection.

Decontamination procedures

Two methods of faecal decontamination were compared. Method 1 was the NaOH/oxalic acid treatment of Beerwerth (1967). Two grams of faeces were homogenised in 50 ml of 4.0% NaOH. The mixture was transferred into a sterile 50 ml polycarbonate screw-capped tube and shaken for 10 min on a horizontal shaker (Bühler, Hechingen, Germany). After centrifugation (3,000 x g, 15 min, 20°C) the pellet was resuspended in 20 ml of 5.0% oxalic acid by thorough repeated agitation on a vortexer and was then shaken for 15 min on a horizontal shaker. The suspension was centrifuged as before, the pellet was resuspended in 4 ml of sterile saline (0.15 M NaCl) and used as inoculum. 200 µl aliquots were transferred to each of three slants of Loewenstein-Jensen (LJ) medium containing 2.0 mg/l mycobactin J and to one slant of LJ medium without mycobactin (Enclit, Oelzschau, Germany). The inoculation of LJ slants was performed as described elsewhere (Kalis et al., 1999).

Method 2 was a hexadecylpyridinium chloride (HPC) decontamination combined with antimicrobial agent treatment adopted from Whitlock and Rosenberger (1990) and Shin (1989). Two grams of faeces were homogenised in 5 ml of 0.75% HPC (Sigma, Buchs, Switzerland). The mixture was transferred into a sterile 10 ml polycarbonate screw-capped tube and incubated for 18 h at ambient temperature. The tube was centrifuged (900 x g, 30 min, 20°C) and the pellet was resuspended in 2 ml of 0.1 M phosphate buffered saline (PBS, 136.9 mM NaCl, 1.46 mM KH₂PO₄, 8.1 mM Na₂HPO₄ × 2H₂O, 2.7 mM KCl, pH 7.4) with 100 µg/ml vancomycin, 100 µg/ml naladixic acid and 50 µg/ml amphotericin B (all purchased from Sigma). After a further 18 h' incubation the suspension was used to inoculate slants as described above.

Culture of mycobacteria

Inoculated tubes were incubated at 37°C for 16 weeks and growth was determined visually at weekly intervals. Culture tubes and samples were recorded as contaminated according to the criteria of Kalis et al. (2000). All bacterial colonies grown on LJ-agar slants were subjected to acid-fast staining by the Ziehl-Neelsen-technique (Ziehl, 1882; Neelsen, 1885). They were examined for acid-fast staining bacteria (AFB) by light microscopy under oil immersion (x1,000). AFB were subcultured on LJ slants with and without mycobactin. MAP was diagnosed on the basis of (i) colony morphology (small, smooth to slightly rough, opaque

to whitish colonies), (ii) micromorphology (acid-fast staining, small, rod-shaped bacteria), and (iii) mycobactin dependency. Pure cultures of MAP as well as mycobactin independent mycobacteria (further referred to as atypical mycobacteria, AM) were propagated in Middlebrook 7H9 broth with ADC-enrichment (Becton Dickinson, Basel, Switzerland) with and without 2.0 mg/l mycobactin J, respectively (Synbiotics Europe SAS, Munich, Germany). Harvests of pure cultures in Middlebrook 7H9 medium containing 50% glycerol (Sigma) were stored at -70°C .

PCR-RFLP analysis

PCR-RFLP analysis was performed for a total of 160 mycobacterial isolates including 15 isolates phenotypically classified as MAP. The following reference strains were used: MAP strain ATCC 19698, MAP strain 6783 (bovine strain, Institute of Microbiology and Animal Infectious Diseases, Veterinary School, Hannover, Germany), *M. avium* subspecies *avium* ATCC 25291. Total DNA was prepared from mycobacterial stocks by a previously described protocol (Hoelzle et al., 2000). MAP was identified by PCR-amplification of the IS1311 insertion sequence and subsequent analysis of the MAP-specific IS1311 RFLP pattern as described by Marsh et al. (1999). For this, a 608-bp fragment amplified from the IS1311 insertion sequence of *M. avium* by using primers M56 (5'-GCGTGAG-GCTCTGTGGTGAA-3') and M119 (5'-ATGAC-GACCGCTTGGGAGAC-3') was digested with restriction endonucleases *Mse*I and *Hinf*I (Bioconcept, Allschwil, Switzerland). For nucleotide sequences analysis amplification products were purified with the QIAQuick PCR Purification Kit (Quiagen, Hilden, Germany). Sequencing was performed by MWG Biotech (Ebersberg, Germany).

Serum antibody ELISA

A commercial ELISA for the detection of antibodies against the MAP-lipoarabinomannan was used (Paratuberculosis ELISA Svanovir[®], Svanova Biotech, Uppsala, Sweden). The test was performed and results were interpreted according to the manufacturer's instructions with sera being tested in a 1:100-dilution in duplicate. Positive and negative control sera were included in every test. Optical densities (OD) were recorded at 450 nm by a computer-aided microplate reader (Tecan, Switzerland).

Results

Efficacy of decontamination protocols

A total of 286 individual faecal samples were decontaminated by two different methods (method 1,

NaOH-oxalic acid; method 2, HPC-antibiotics), and were subsequently cultured on four slants of LJ medium each resulting in a total of 1144 cultures for each decontamination protocol. Of the 1144 method-1 cultures, 56 (4.9%) were contaminated after 4 weeks of incubation, increasing cumulatively to 100 (8.7%), 141 (12.3%), and 202 (17.6%) after 8, 12 and 16 weeks of incubation respectively. For 9 samples (3.1%) all 4 tubes were lost due to bacterial overgrowth. By comparison the method-2 decontamination resulted in 150 tubes (13.1%) with overgrowth of bacterial contaminants after four weeks of incubation and increasing cumulatively to 197 (17.2%), 221 (19.3%), and 246 (21.5%) after 8, 12 and 16 weeks respectively. 21 samples (7.3%) were lost completely. The rate of contaminated culture tubes and the number of samples lost due to contamination was significantly higher in faecal samples treated with method 2 ($p < 0.01$, McNemar's test). Only of one faecal sample (0.4%) all tubes from both decontamination protocols were lost due to microbial overgrowth.

Culture of mycobacteria

MAP was grown after 4 to 16 weeks of incubation from 15 of 186 animals (8.1%) from herds with a history of paratuberculosis (group A). MAP was grown from at least one animal of each group A herd. All 15 MAP isolates were obtained from samples treated with method 1 (8.1%) whereas only 3 MAP isolates (1.6%) were obtained from samples treated with method 2. MAP was not isolated from faeces of group B animals. The detection rate of MAP was significantly higher in samples treated with method 1 ($p < 0.01$, McNemar's test). Atypical mycobacteria (AM) were grown from 86 animals of group A (46.2%) and from 48 animals of group B (48.0%). The overall detection rate of AM was 46.8% (134 of 286 animals). All AM were grown only from faecal samples treated with method 1. Ten samples revealed growth of two and one sample revealed growth of three different AM colony morphotypes. Pure cultures were established from a total of 145 AM.

PCR-RFLP analysis

A total of 160 mycobacterial isolates (15 MAP, 145 AM) were examined by PCR for the presence of the *M. avium*-specific IS1311 insertion element. A PCR amplification product of the predicted size of 608 bp was obtained from *M. avium* reference strains and from 15 isolates which had been classified phenotypically as MAP before. The RFLP profile of the IS1311-PCR products of all these isolates exhibited 4 fragments of 323, 285, 218, and 67 bp which are characteristic for the bovine type of MAP (Fig. 1). In comparison, the *M. avium* subspecies *avium* reference strain

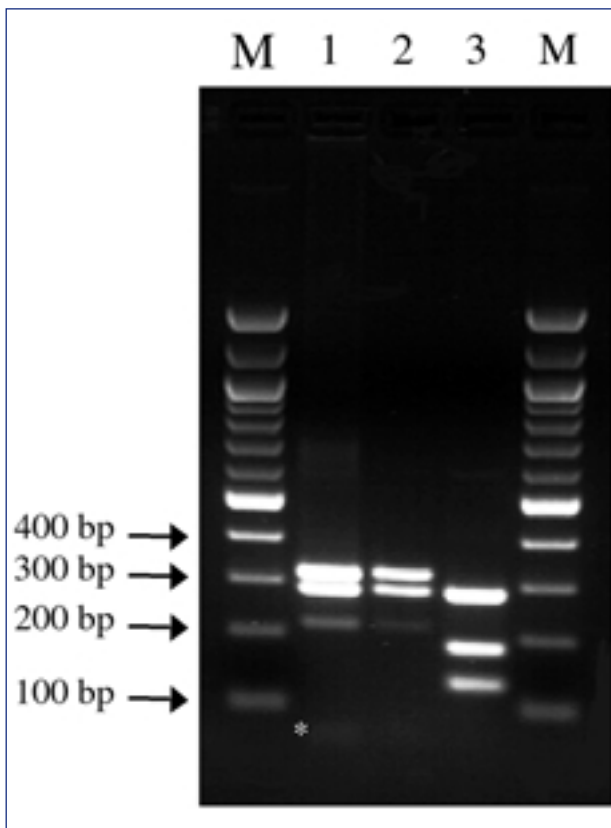


Figure 1: *MseI/HinfI* RFLP analysis of the *IS1311* insertion element of *Mycobacterium avium*. Lane M, DNA size marker (100-bp DNA ladder); lane 1, *M. avium* subsp. *paratuberculosis* ATCC 19698; lane 2, *M. avium* subsp. *paratuberculosis* bovine faecal isolate; lane 3, *M. avium* subsp. *avium* ATCC25291; *, the 67-bp band is difficult to detect after electrophoresis in 3% agarose gels.

displayed a clearly distinct pattern consisting of 3 fragments of 285, 189, and 134 bp. Nucleotide sequences of *IS1311*-PCR products were 99.9 to 100% identical with data base entries (GenBank acc. no. U16276).

Serum antibody ELISA

The test validity criteria were fulfilled in all ELISA tests performed in our study (OD pos. control >1.00, OD neg. control <10% of OD pos. control). Based on the manufacturer's interpretation criteria animals were classified "positive", "doubtful" or "negative" as shown

in table 1. Positive ELISA reactions indicating the presence of MAP serum antibodies were detected in 39 of 186 animals (20.9%) from dairy herds with a history of paratuberculosis (group A). MAP was grown from 6 of the 39 seropositives (15.4%). A sample of 15 sera (8.0%) revealed uncertain ELISA results. MAP was cultured from 2 animals with a doubtful serology (13.3%) and from 7 animals with a negative serology (5.3%). Positive and doubtful ELISA results were found in 9 and 8 animals, respectively from group B (9.0%, 8.0%). Culture of MAP was negative for all animals of group B.

Discussion

A broad variety of methods employed for eliminating bacterial and fungal contaminants from bovine faecal specimens prior to cultural recovery of MAP has been evaluated with most laboratories relying on the use of the NaOH/oxalic acid or the HPC decontamination (Merkal et al., 1982; Kalis et al., 1999). In the present study the HPC method adopted from Whitlock and Rosenberger (1990) and Shin (1989) resulted in a significant lower yield of MAP and a significant higher rate of contaminated culture tubes than the NaOH/oxalic acid method. Moreover, atypical mycobacteria (AM) were grown from 46.8% of NaOH/oxalic acid treated specimens but were not obtained from any HPC-treated sample. Here our results support the assumption that NaOH/oxalic acid decontamination is more appropriate than HPC to preserve the viability of mycobacteria including MAP from bovine faeces. The unsatisfactory performance of the HPC method contradicts evidence from the literature which is, however, admittedly limited. Besides the studies of Kenefick et al. (1988) and Stachelscheid (1989), systematic investigations on the effect of HPC on MAP-viability in bovine faeces in comparison with other chemical treatments have not been published. In both said studies treatment of faecal samples with HPC is suggested less detrimental to MAP than NaOH/oxalic acid. Our results are in dispute with those studies. Interplay of a variety of factors may contribute to the outcome of the decontamination pro-

Table 1: Comparison of culture and serology.

Culture	Humoral immune response measured by ELISA					
	Group A (n = 186) ¹			Group B (n = 100) ²		
	positive	equivocal	negative	positive	equivocal	negative
MAP ³	3.2 ⁵	1.1	3.8	0	0	0
AM ⁴	9.1	2.7	33.9	1.0	5.0	42.0
no mycobacteria	8.6	4.3	33.3	8.0	3.0	41.0
Total	20.9	8.1	71.0	9.0	8.0	83.0

¹ samples from herds with a history of clinical paratuberculosis, ² samples from herds without a history of paratuberculosis, ³ *M. avium* ssp. *paratuberculosis*, ⁴ AM: atypical mycobacteria, ⁵ results in %.

cedure and further studies should help clarify these discrepancies. At any rate the NaOH/oxalic acid method was useful to detect MAP in all 10 herds with a history of paratuberculosis. Since the detection of a single infected animal is sufficient to classify a dairy herd as infected, our study confirmed the reported applicability of the NaOH/oxalic acid method as a suitable tool to screen dairy stocks for intestinal MAP infections by culture (Kalis et al., 2000; Muskens et al., 2003). In addition, PCR-RFLP analysis of the IS1311 insertion element offers a valuable method to identify MAP.

In paratuberculosis control programs culture of MAP and serodiagnosis should be performed concurrently. Since the strength of the antibody response depends directly on the disease progress, results of faecal culture and serology are not necessarily complementary. Sensitivity of serology, i.e. ELISA is high in clinically affected animals (Collins and Sockett, 1993; Sweeney et al., 1995; Whitlock et al., 2000) but 63 to 91% of asymptomatic cattle with a culture-confirmed paucibacillary faecal shedding of MAP remain unidentified as infected by serology (Eamens et al., 2000; Muskens et al., 2003). This was also observed in our study: four animals with clinical paratuberculosis revealed a strong positive ELISA reactivity. Out of the remaining 11 MAP-culture positive cows without clinical symptoms only six (40.0%) exhibited a positive antibody response, thus corroborating the low sensitivity of serology in subclinically infected cows. By repeated serological testing over nine months, seroconversion has been observed in approximately 64% of formerly seronegative MAP-shedding cattle (Eamens et al., 2000). Thus the lack of reactivity in sera from MAP shedders tends to represent animals prior to the development of a significant humoral immune response. Out of the 186 cows from herds with a history of paratuberculosis 39 (20.9%) were seropositive; this percentage was significantly higher than in the group of arbitrarily selected 100 cows representing 100 dairy herds without any history of paratuberculosis mainly from the eastern part of Switzerland (9.0%). Out of the 39 seropositives from affected herds only six cows (15.4%) tested culture-positive. This is also reflected in the study from the Netherlands of Muskens et al. (2003) who found 17.3% of 371 seropositive cows culture-positive. The positive and negative predictive values of the ELISA used in our study are high (Jark et al., 1997). Therefore, it seems reasonable to consider our serologic results as true, and we agree with other authors who stated that only a low percentage of ELISA-positive cattle can be confirmed

as MAP shedders by faecal culture mainly due to intermittent shedding of varying numbers of MAP (Muskens et al., 2003). Nevertheless, it is tempting to assume that false-positive ELISA results may to some degree cause the divergence between serology and culture especially in view of the high percentage of animals with a faecal shedding of AM. Infection of cattle with AM, e.g. *M. kansasii* can evoke an immune response with highly cross-reactive mycobacterial antibodies (Schliesser, 1965; Kazda, 1969). Antibodies against AM are known as a cause of false-positive reactions in MAP serology but can be avoided by absorption of sera with *M. phlei* (Yokomizo et al., 1985). The ELISA test used in our study was performed with unabsorbed sera as recommended by the manufacturer. Interestingly, the presence of AM in faeces as confirmed by microscopy and culture was not correlated with any increased ELISA reactivity (data not shown). These results indirectly provide evidence for the validity of positive ELISA reactivities observed in animals without a MAP-positive faecal culture.

Since the work of Beerwerth (1967) who isolated AM from 86.0% of 401 bovine faecal specimens, it is well recognized that the bovine intestinal tract harbours a broad variety of AM which are usually transient microbes from environmental sources. Our findings of faecal shedding of AM in 46.8% of animals tested is of interest for further studies on the prevalence of the IS900 insertion element in mycobacteria unrelated to MAP. IS900 is considered unique to MAP and is, therefore, used as target for the PCR-based diagnosis of MAP from bovine clinical specimens like faeces or milk (Erume et al., 2001; Corti and Stephan, 2002; Halldorsdottir et al., 2002.) Recently, environmental mycobacteria have been shown to harbour IS900-like elements which are amplifiable with primers designed for detection of the MAP-IS900 element (Cousins et al., 1999; Englund et al., 2002; Bull et al., 2003). Since PCR amplicons from known IS900-like elements did not differ in size from IS900 elements, specificity of IS900-PCR products needs to be substantiated by amplicon sequencing. It would be important to test our strain collection of AM for the presence of IS900 analogs using methodologies such as those described by others (Cousins et al., 1999; Englund et al., 2002).

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Mise en évidence par culture et sérologie de *Mycobacterium avium* subspecies *paratuberculosis* chez des vaches laitières

Des échantillons de selles provenant de 186 vaches issues de 10 exploitations avec des cas cliniques sporadiques de paratuberculose (groupe A) ainsi que des échantillons provenant de 100 vaches issues d'exploitations sans anamnèse de paratuberculose ont été examinés par culture quant à la présence de *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Les échantillons ont été décontaminés parallèlement avec NaOH/acide oxalique et avec du chlorure d'hexadecylpyridinium (HPC) et cultivé sur Agar oblique de Loewenstein-Jensen avec ou sans mycobactine. Les cultures ont été incubées durant 16 semaines et examinés quant à la présence de bactéries acido-résistantes. Le MAP a été identifié sur la base de sa dépendance à la mycobactine et par analyse PCR-RFLP de l'élément d'insertion IS1311. Le MAP a été isolé des selles de 15 animaux du groupe A (8.1%) mais dans aucun échantillon du groupe B. Dans les échantillons décontaminés au moyen de NaOH/acide oxalique la mise en évidence de MAP a été nettement plus haute (8.1% contre 1.6%) et le taux de cultures contaminées significativement plus bas (17.6% contre 21.5%) que dans les échantillons décontaminés au HPC. Des mycobactériacées atypiques n'ont pas pu être mis en évidence dans les cultures à base de selles décontaminées au HPC; elles ont par contre été isolées dans 48.6% des cultures issues de selles traitées au NaOH/acide oxalique. Dans un test Elisa utilisant comme antigène le lipoarabinomannane du MAP, 16.8% des 286 vaches présentaient des anticorps contre le MAP.

Esame colturale e sierologico di *Mycobacterium avium* subspecies *paratuberculosis* nelle mucche da latte svizzere

La presenza di *Mycobacterium avium* subspecies *paratuberculosis* (MAP) è stata esaminata in coltura su campioni di feci di 186 mucche di 10 mandrie con manifestazioni cliniche sporadiche di paratubercolosi (gruppo A) e di 100 mucche di mandrie senza indicazioni anamnestiche di paratubercolosi. I campioni di feci sono stati decontaminati parallelamente con NaOH/acido ossalico e con 0.75% di cetylpyridinium chloride (HPC) e con l'impiego di agar di Loewenstein-Jensen addizionato con mycobactin o senza. Le colture sono state incubate per 16 settimane e esaminate sulla crescita di batteri acido resistenti. Il MAP è stato identificato grazie alla dipendenza al mycobactin e tramite analisi PCR-RFLP dell'elemento inserito IS1311. Il MAP è stato identificato sulle feci di 15 animali del gruppo A (8.1%) mentre nelle feci in coltura del gruppo B non era identificabile. Nei campioni di feci decontaminati in NaOH/acido ossalico la percentuale dimostrata di MAP era chiaramente più alta (8.1% vs. 1.6%) e la percentuale in coltura contaminata era chiaramente inferiore (17.6% vs. 21.5%) che nei campioni di feci decontaminati con HPC ($p < 0.01$, test McNemar's). La presenza di micobatteri atipici non è stata rilevata nelle colture di campioni di feci decontaminati con HPC mentre, sono state isolate nel 46.8% dei campioni di feci trattati con NaOH/acido ossalico. In un test commerciale ELISA con lipoarabinomannan MAP in quanto antigene sono stati rilevati in 16.8% delle 286 mucche anticorpi contro MAP. In seguito l'esame colturale e sierologico ha portato nel 15.4% dei casi risultati positivi conformi.

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