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Utilisation of Actiphage in combination with IS*900* qPCR as a diagnostic tool for rapid determination of paratuberculosis infection status in small ruminant herds

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Abstract

Introduction: *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis, a chronic infectious intestinal disease occurring in domestic and wild ruminants. Early diagnosis of infected herds enabling timely adoption of control measures is tremendously important in view of the fact that the disease has a significant economic impact on farmers. The aim of this study was to evaluate the possibility of rapid detection of viable MAP on small ruminant farms based on environmental sample examination using a novel phage-based test named Actiphage. **Material and Methods:** A total of 9 fresh and 28 frozen (8 or 11 years at −70°C) environmental samples originating from paratuberculosis-affected farms were analysed for the presence of MAP by four different diagnostic methods: Actiphage combined with real-time PCR targeting insertion sequence *900* (IS*900* qPCR), conventional phage amplification assay, culture (frozen samples only), and direct IS*900* qPCR. **Results:** Viable MAP was detected in one fresh environmental sample using Actiphage–IS*900* qPCR. None of the frozen samples tested positive using this diagnostic approach, which was consistent with the results of culture examination also providing information on viability. **Conclusion:** This study describes other possible and innovative uses of phage-based methods in paratuberculosis control strategies. The Actiphage-qPCR was found to be less laborious than culture and provided results within six hours, suggesting that it may be a valuable tool for rapid initial determination of the infectious status of farmed animals based on environmental sample examination.

Keywords: Actiphage-qPCR, environmental samples, *Mycobacterium avium* subsp. *paratuberculosis*, phage amplification assay, small ruminants.

Introduction

Paratuberculosis, also known as Johne's disease, is a chronic and fatal inflammatory intestinal disease caused by the slow-growing acid-fast pathogen *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The disease mainly affects domestic and wild ruminants and results in decreases in milk and meat production leading to substantial financial losses worldwide (6, 13). The shedding of MAP in the faeces of infected individuals contributes to environmental contamination, and there is a high risk of MAP transmission to other healthy farmed animals by the faecal–oral route in herds that are structured in groups in which the animals share an environment (24). The level of calves' exposure to an environment contaminated by infected adults and the hygiene measures adopted were found to be pivotal in MAP transmission (12).

One of the key issues that complicates the control of paratuberculosis on farms is the resistant nature of MAP and its ability to survive in the environment for a long time. MAP was found to remain viable after 55 weeks in soil mixed with manure and to persist in the farm environment even after the depopulation of infected herds (23). Studies focusing on the circulation of MAP on farms reported that it could spread through faeces, dust, aerosol or plants (1, 5, 23). Environmental samples are becoming important in the diagnosis of paratuberculosis in pasture-bred cattle and other paratuberculosis-susceptible ruminants. This type of sample is, therefore, among the matrices that are examined in paratuberculosis control and monitoring

programmes and represents a reliable indicator of the infection status of herds (7, 25). A standardised protocol for MAP detection based on the cultivation of environmental samples was found to have herd-level sensitivity comparable to those of ELISA and pooled faecal culture (3). In addition, the results of a simulation study suggested that MAP culture of environmental samples was the most cost-effective method for initial classification of the infection status of herds with low, moderate and high MAP prevalence (21).

Although early identification of infected herds is a prerequisite for the control of disease, diagnosis is hampered by the long incubation periods of MAP, variable immune response, the inability of some tests to make live-dead differentiation and the insufficient sensitivity of diagnostic tests, such as cultivation, real-time PCR (qPCR) and ELISA (4). One of the promising new diagnostic approaches, which has the potential to overcome the shortcomings of conventional tests and which provides a quick result, is a technique based on the use of lytic bacteriophage D29 to release mycobacterial DNA, which is subsequently detected and quantified by qPCR (20). A test applying this bacteriophage is currently commercially available under the name Actiphage (PBD Biotech, Birmingham, UK) and in combination with qPCR targeting insertion sequence *900* (IS*900*) allows viable MAP cells to be detected in a substantially shorter time (six hours) than when using conventional culture techniques (six weeks or more) and with significantly higher sensitivity (a limit of detection ≤10 cells/mL of blood) (20). This test has been shown to be effective in detecting MAP or *M. tuberculosis* complex bacteraemia in both cattle and human patients (2, 20, 22).

The aim of the present study was to evaluate the applicability of the recently developed phage-based assay named Actiphage in combination with IS*900* qPCR on environmental samples obtained from paratuberculosis-affected small ruminant farms and to explore the possibility of using this tool to determine the infectious status of herds. Both fresh and long-term frozen environmental samples were examined for the presence of viable MAP by Actiphage–IS*900* qPCR. Comparisons were made with the results of direct IS*900* qPCR, culture (frozen samples only) and a conventional phage amplification assay (PA). A further goal was to test the specificity of phage D29 (supplied in the Actiphage Rapid kit) using selected mycobacterial species occurring in the environment that may cross-react in an ELISA test and give false positive signals.

Material and Methods

Sample collection and processing. Environmental samples (fresh and frozen) were collected on two paratuberculosis-susceptible animal farms with a known history of paratuberculosis. At the time of sampling, one farm numbered 40 sheep in breeding with serum-ELISA and/or IS*900* qPCR-positive animals present. The second farm comprised a total of 72 animals (60 head of mouflon and 12 head of fallow deer) with clinical signs of paratuberculosis. One group of nine samples was analysed immediately, the second group of 28 samples was frozen for 8 or 11 years at −70°C. Both groups of samples were tested by conventional PA and Actiphage-IS*900* qPCR (Tables 1 and 2). The results were compared with those of a direct IS*900* qPCR and culture (frozen samples only), which were performed immediately after sample collection.

The processing of environmental samples drew on the method of an earlier study using faecal samples (9). Briefly, 5 g of the total environmental sample was homogenised in 30 mL of sterile distilled water on a vortex and subsequently centrifuged at 100 × *g* for 30 s. Then, 1.5 mL of supernatant was collected in triplicate in a screw-cap tube and centrifuged at 11,300 × *g* for 10 min. The pellets were examined for the presence of MAP using direct IS*900* qPCR, conventional PA, and Actiphage–IS*900* qPCR. Fresh sample no. 5 (cobweb) was processed with only 0.25 g of the sample being investigated by these three tests. For the examination of sample no. 5 by conventional PA and Actiphage–IS*900* qPCR, 2 × 0.25 g of the sample was homogenised in 1 mL of sterile distilled water on a vortex and subsequently centrifuged at 100 × *g* for 30 s. The supernatant was collected and centrifuged at 11,300 × *g* for 10 min, and the pellet was used for analyses.

Table 1. Examination of fresh environmental samples for the presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) using an Actiphage–IS*900* qPCR assay, direct IS*900* qPCR, and a conventional phage amplification assay (PA)

Sample no.	Sample characteristics	Direct IS*900* qPCR (copy/g)	Conventional PA (PFU/g)*	Actiphage–IS*900* qPCR (copy/g)
1	Soil	5.42×10^2	4.80×10^2	–
2	Soil	1.09×10^2	2.18×10^2	–
3	Soil	1.02×10^2	2.88×10^2	–
4	Soil	4.20×10^2	–	–
5	Cobweb	1.20×10^2	–	–
6	Soil/plants	1.64×10^0	7.54×10^3	1.84×10^0
7	Soil	5.88×10^0	3.06×10^2	–
8	Soil	6.56×10^0	1.09×10^2	–
9	Soil	2.40×10^0	4.36×10^1	–

PFU – plaque-forming units; – denotes a negative result; * – plaques occurred during the conventional PA, but MAP was not confirmed by plaque PCR in any of the samples (five plaques per sample were tested)

Table 2. Examination of frozen environmental samples for the presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) using an Actiphage–IS*900* qPCR assay, direct IS*900* qPCR, a conventional phage amplification assay (PA) and culture

Sample no.	Sample characteristics	Time frozen	Direct IS*900* qPCR (copy/g)*	Conventional PA (PFU/g)†	Culture*	Actiphage–IS*900* qPCR
1	Soil	11 years	6.43×10^3	6.98×10^2	–	–
2	Soil	11 years	1.18×10^3	8.72×10^2	–	–
3	Soil	11 years	1.16×10^2	2.88×10^3	–	–
4	Soil	11 years	2.11×10^2	1.92×10^3	–	–
5	Soil	11 years	1.30×10^2	2.01×10^2	–	–
6	Soil	11 years	7.94×10^2	2.62×10^2	–	–
7	Soil	11 years	3.48×10^2	7.85×10^2	–	–
8	Roots	11 years	1.07×10^2	3.49×10^2	–	–
9	Soil	11 years	7.39×10^2	2.62×10^2	–	–
10	Roots	11 years	1.68×10^2	3.49×10^2	–	–
11	Soil	11 years	1.61×10^2	2.62×10^2	–	–
12	Roots	11 years	4.36×10^2	6.98×10^2	–	–
13	Roots	11 years	6.41×10^2	2.27×10^2	–	–
14	Soil	11 years	9.81×10^2	6.10×10^2	–	–
15	Soil	11 years	4.09×10^2	1.74×10^2	–	–
16	Roots	11 years	1.57×10^2	5.58×10^2	–	–
17	Soil	8 years	4.40×10^2	4.36×10^2	–	–
18	Detritus	8 years	1.25×10^2	4.36×10^1	–	–
19	Feed residues	8 years	1.94×10^2	–	–	–
20	Soil	11 years	2.67×10^1	8.72×10^1	–	–
21	Soil	11 years	7.26×10^1	1.13×10^3	–	–
22	Soil	11 years	8.93×10^1	5.67×10^2	–	–
23	Soil	11 years	6.11×10^1	3.49×10^3	–	–
24	Soil	11 years	7.95×10^1	1.74×10^2	–	–
25	Roots	11 years	6.44×10^1	5.23×10^1	–	–
26	Soil	11 years	7.90×10^1	8.72×10^0	–	–
27	Soil	11 years	2.70×10^1	1.40×10^2	–	–
28	Roots	11 years	9.91×10^1	1.74×10^2	–	–

PFU – plaque-forming units; – denotes a negative result; * – the analysis was performed immediately after sample collection; † – plaques occurred during the conventional PA, but MAP was not confirmed by plaque PCR in any of the samples (five plaques per sample were tested)

Culture. Culture examination of environmental samples was performed as described previously on Herrold's egg-yolk medium (HEYM) supplemented with 2 mg/mL Mycobactin J (Allied Monitor, Fayette, MO, USA) after the samples had been decontaminated with hexadecyl pyridinium chloride (N-cetylpyridinium chloride monohydrate; Merck, Darmstadt, Germany) (9).

Conventional phage amplification assay. The conventional PA was performed as described earlier (6) with minor modifications. In brief, the pellet was resuspended in 1 mL of Middlebrook 7H9 broth (M7H9; Difco Laboratories, Detroit, MI, USA) supplemented with 10% Middlebrook OADC enrichment (Difco Laboratories), 2 mM $CaCl_2$ (Penta Chemicals, Prague, Czech Republic) and 1.25% PANTA antibiotic mixture (Becton Dickinson, Franklin Lakes, NJ, USA). The antibiotic mixture was prepared according to the manufacturer's instructions and used to suppress the growth of other bacteria present in the samples. Following overnight incubation at 37°C with shaking at 100 rpm, 100 µL of bacteriophage D29 (stock concentration 10^9 plaque-forming units (PFU)/mL) was added and the sample was incubated at 37°C for 2 h with shaking at 100 rpm. Then, 100 µL of 100 mM ferrous ammonium sulphate solution (FAS; Lach-Ner, Neratovice, Czech Republic) was added and the samples were incubated at room temperature for 5 min, followed by FAS neutralisation by the addition of 5 mL of enriched medium and preparation of tenfold serial dilutions of samples. A 1 mL aliquot of *Mycobacterium smegmatis* mc^2155 (10^8 colony-forming units (CFU)/mL), grown for 2 days at 37°C with shaking at 100 rpm in 50 mL of M7H9 broth with 10% oleic albumin dextrose catalase (OADC), was added to each sample dilution. Each of the samples was transferred into a sterile Petri dish and 6 mL of molten 1.6% Middlebrook 7H10 agar (Difco Laboratories) cooled to 55°C was added. The content of the plate was mixed and left at room temperature until the agar set. The number of plaques formed was counted after overnight incubation of the plates at 37°C.

The identity of the mycobacteria detected was analysed by plaque PCR (19) with five plaques per sample being examined. The centre of the plaque was excised using a sterile loop and transferred to 10 µL of ultrapure water (Top-Bio, Vestec, Czech Republic), heated at 95°C until the agar melted, and immediately frozen at −20°C. After 15 min at this temperature, the samples were thawed and the DNA present in the supernatant was analysed by an in-house IS*900* qPCR (18).

Actiphage assay and purification of mycobacterial DNA. The extraction of MAP DNA using an Actiphage Rapid Kit (PBD Biotech) was performed according to the manufacturer's instructions, after which it was purified using the PurePhage Kit protocol (BioSellal, Dardilly, France). For frozen environmental samples, before performing the Actiphage-qPCR assay, pellets were resuspended in 1 mL of Actiphage Medium Plus (PBD Biotech), incubated overnight at 37°C and centrifuged at 13,000 × *g* for 3 min to recover MAP. Then the supernatant was removed.

Isolation of MAP DNA. Examination of fresh sample no. 5 by direct IS*900* qPCR followed DNA isolation respecting the protocol of the PowerSoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA), with 0.25 g of sample being examined. Isolation of the DNA from the rest of the samples was based on a slightly modified protocol from the Quick-DNA Fecal/Soil Microbe Microprep Kit (Zymo Research, Irvine, CA, USA). Briefly, the pellet was dissolved in 750 µL of BashingBead Buffer and transferred to a BashingBead Lysis Tube. The sample was then subjected to mechanical homogenisation in a MagNA Lyser (Roche Diagnostics, Mannheim, Germany) at 6,400 rpm for 1 min. Following centrifugation at 10,000 × *g* for 1 min, 400 µL of supernatant was processed using the isolation kit according to the manufacturer's instructions. Isolated DNA was eluted into 25 µL of DNA Elution Buffer and used as a template for the IS*900* qPCR.

Amplification of the IS*900* signature sequence. The detection and quantification of MAP cells by IS*900* qPCR was performed using a LightCycler 480 instrument (Roche Diagnostics) with 5 µL of isolated DNA in technical duplicates used as a template (18).

Specificity of bacteriophage D29. In order to investigate the possibility of detecting other mycobacteria that are naturally prevalent in the environment, two-week-old cultures of *M. kansasii* (43G6) and *M. avium* subsp. *avium* (43/860) cultured on HEYM at 37°C were analysed using Actiphage-qPCR and the results were compared with those obtained on MAP serving as a positive control. Colonies of all three cultures were resuspended in M7H9 broth with OADC enrichment and 2 mM $CaCl_2$, diluted to $OD_{600} \approx 0.15–0.20$ and further diluted tenfold three times. Two 1 mL aliquots of all diluted suspensions were centrifuged at 13,000 × *g* for 3 min to recover mycobacterial cells and then processed by the Actiphage assay and qPCR targeting the ITS locus for both *M. kansasii* and *M. avium* subsp. *avium* (16) and IS*900* for MAP (18).

Results

Fresh environmental sample testing by Actiphage–IS*900* qPCR. Of the 9 fresh environmental samples, 1 (11.11%) tested positive by Actiphage-qPCR with an estimated viable MAP quantity of 1.84×10^0 cells/g of sample (Table 1). In contrast, conventional PA detected viable mycobacteria in 7 (77.78%) samples examined (including the Actiphage-qPCR–positive sample) with mycobacterial cell quantities ranging from 4.36×10^1 to 7.54×10^3 PFU/g of sample, although none of these samples gave a positive result by plaque PCR. Regarding the direct IS*900* qPCR, MAP DNA was revealed in all samples tested, with quantity estimates ranging from 1.64×10^0 to 5.42×10^2 copies/g of sample.

Frozen environmental sample testing by Actiphage–IS*900* qPCR. All 28 samples were found to be positive using direct IS*900* qPCR (range: 2.67×10^1 – 6.43×10^3 MAP cells/g) when freshly collected, but were negative by culture (Table 2). Following long-term freezing for 8 or 11 years, none of the examined samples gave a positive result using Actiphage-qPCR. Consistent culture and Actiphage-qPCR results indicate that no viable MAP cells were present in the samples. On the other hand, plaques were produced in all but one sample (96.43%) when using conventional PA as a diagnostic tool (ranging from 4.36×10^1 to 3.49×10^3 PFU/g); however, none of these gave a positive plaque PCR result (with internal amplification control providing a clear signal excluding qPCR inhibition) with five plaques per sample being analysed. Although the possible presence of viable MAP cells in these specimens cannot be excluded because not all plaques formed on an agar plate were tested, it can be concluded from the results that environmental mycobacteria other than MAP were present in these samples.

Specificity of bacteriophage D29. Considering that numerous samples produced plaques on an agar plate but without a positive result by plaque PCR, it was concluded that environmental mycobacteria other than MAP were most likely present in these samples. Therefore, in order to verify the susceptibility of other mycobacterial strains to bacteriophage D29 infection and to evaluate the possibility of detecting mycobacteria other than MAP by Actiphage-qPCR assay, pure cultures of *M. kansasii* and *M. avium* subsp. *avium* strains were subjected to testing with Actiphage. Using this test in combination with qPCR targeting the ITS sequence of DNA, which is present in all members of the *Mycobacterium* genus, it was possible to detect both strains analysed, indicating that bacteriophage D29 is capable of infecting both *M. kansasii* and *M. avium* subsp. *avium* cells.

Discussion

The aim of this study was to evaluate the applicability of the recently developed Actiphage assay combined with IS*900* qPCR to examine naturally contaminated environmental samples for the presence of viable MAP cells. To date, no study has been conducted on the use of phage-based assays to selectively detect viable MAP in samples originating from the farm environment. Phage-based methods providing information

on viability have great potential in the early diagnosis of mycobacterial infections, especially in paratuberculosis (14), and could serve as a means for rapid initial determination of the infectious status of farmed animals based on environmental sample examination. Methods based on PCR are commonly used for diagnostic and monitoring purposes (1, 11), although they cannot differentiate between viable and dead cells and are therefore unable to distinguish between active and passive infection of animals (10). On the other hand, the application of cultivation methods enabling viability determination is substantially hindered by the very long incubation times of MAP cells (17). The recently developed phage-based assay named Actiphage, in combination with IS*900* qPCR, overcomes the shortcomings of the conventional diagnostic tests (14, 20) and may, therefore, represent an effective tool for paratuberculosis control strategies in farmed animals.

Using Actiphage–IS*900* qPCR, the presence of viable MAP was demonstrated in 1 of 9 fresh environmental samples originating from a paratuberculosis-affected farm (Table 1). The MAP load determined in this sample corresponded to the MAP quantity obtained by direct IS*900* qPCR (units of cells/g). In addition, this sample provided the highest value of viable mycobacteria quantity using conventional PA. Although we tested only one sample positively using Actiphage-qPCR, the occurrence of viable MAP in the farm environment cannot be ruled out and may pose a risk of indirect paratuberculosis transmission to susceptible animals on the farm. No positive result was observed in any of the frozen environmental samples tested using Actiphage-qPCR (Table 2). Although it has previously been reported that the freeze–thaw process may have an adverse effect on the viability of mycobacteria and may affect the resulting categorisation of samples as MAP positive or MAP negative (4), all these samples also tested negative by culture (before sample freezing), suggesting that no false-negative results were obtained by the Actiphage-qPCR assay and that the positive result of direct IS*900* qPCR is due to the presence of merely a low quantity of dead MAP cells.

Using conventional PA, many fresh and frozen environmental samples produced plaques on an agar plate (with a few cases presenting PFU quantities exceeding the DNA copy number determined by direct qPCR); however, none of them gave a positive result in the plaque PCR (with five plaques tested per sample). Because we did not test all plaques formed on agar plates, there is a certain probability that some plaques formed due to the presence of viable MAP were missed. However, considering the results of other methods providing information on viability (Actiphage-qPCR and culture), it is very likely that viable MAP was not present in these samples and plaques appeared as a result of bacteriophage D29 replication in environmental mycobacteria other than MAP.

Given the likely presence of environmental mycobacteria other than MAP in the specimens tested, another aim was to determine whether these mycobacteria could also be detected using Actiphage-qPCR. For this purpose, cell suspensions of *M. kansasii* and *M. avium* subsp. *avium*, which occur commonly in the environment (8), were selected and processed by Actiphage in combination with a qPCR targeting sequences present in all members of the *Mycobacterium* genus. Both analysed strains gave a positive Actiphage-qPCR result indicating susceptibility to bacteriophage D29 infection. The ability of bacteriophage D29 to infect a wide range of members within the *Mycobacterium* genus has been described previously, with productive infection observed, for example, in strains of *M. ulcerans*, *M. scrofulaceum* and *M. avium* (15). The successful application of Actiphage technology in detection of these mycobacteria may have relevance for the diagnosis of atypical mycobacterioses in human medicine, although more specific target sequences will need to be used to identify particular species accurately by endpoint qPCR.

This is the first demonstration of the use of phage-based assays to detect and quantify viable MAP cells in environmental samples. Actiphage–IS*900* qPCR was found to be much faster and easier to perform than conventional PA and culture, and the advantages are the possibility of automation of the procedure and high-throughput testing. Although Actiphage-qPCR appears to have potential for paratuberculosis control strategies, further studies involving more environmental samples positive for viable MAP are required to determine the performance characteristics accurately and to validate this test on samples of this type.

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