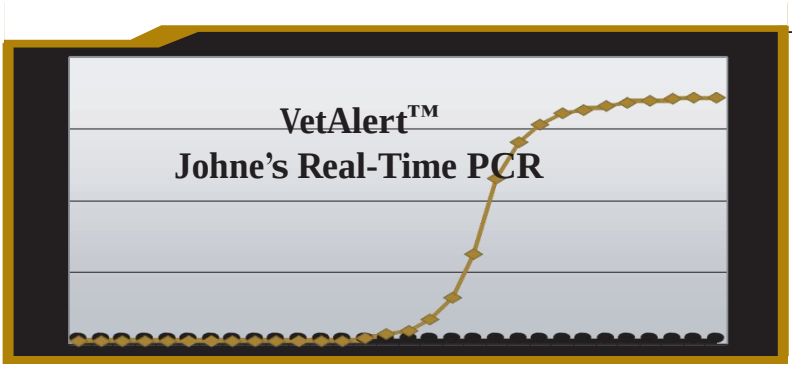
Mycobacterium avium subspecies Paratuberculosis DNA Test Kit, Polymerase Chain Reaction



For qualitative detection of ***Mycobacterium avium*** **subspecies** ***paratuberculosis*** DNA extracted from bovine fecal samples or culture

11 June 2019

Name and Intended Use

The kit is intended as an aid in the *in vitro* qualitative detection of *Mycobacterium avium* subspecies *paratuberculosis* DNA by real-time polymerase chain reaction (PCR). The test can be used to detect DNA extracted directly from bovine fecal samples or from culture media used to amplify the agent in bovine fecal samples. The test is intended as an aid in the diagnosis of Johne's disease in cattle.

Summary and Explanation

Johne's disease is caused by the bacterium *Mycobacterium avium* subspecies *paratuberculosis.* Johne's is a chronic debilitating disease that is a great source of economic loss for the cattle and dairy industry.

The current reference method for diagnosis is culturing of fecal samples. Culturing is time consuming, taking up to three months for a negative result. Recent advances in real-time PCR chemistry have led to improvements in assay sensitivity that allow for both adequate sensitivity and considerably greater speed in the diagnosis of Johne's disease.

Principles of the Procedure

The test is based on real-time PCR that, in addition to specific forward and reverse oligonucleotide primers, utilizes fluorogenic probe hydrolysis chemistry to generate a fluorescent signal when specific *M. avium ssp. paratuberculosis* DNA is present in samples. The primers and probe target the hspX gene, which contains *M. avium ssp. paratuberculosis*-specific DNA sequences that allow for molecular discrimination of *M. avium* ssp. *paratuberculosis* from other closely related *Mycobacterium* species.

Product Description

VetAlert™ Johne's Real-Time PCR TC-9828-100

Materials Provided:

Component	Contents
Mastermix (violet capped tubes)	Real-Time PCR Mastermix, containing primers, FAM-labeled probe, buffers, and *Taq* Polymerase Enzyme; 4 vials/kit
Positive Control (green capped tube)	Oligonucleotide, 1 vial/kit
Product Insert	1/kit

Materials and Equipment Required, but Not Provided:

- MAP Extraction System (Tetracore Cat# TC-9014-100)
- Real-time PCR thermocycler instrument, capable of simultaneously amplifying DNA and detecting a fluorescent signal generated by a TaqMan fluorogenic probe (the instrument should include an optical system which illuminates the reaction vessels and collects the resulting fluorescence emission, a thermal cycler, and data acquisition and analysis software, e.g. ABI 7500, BioRad iCycler iQ®, Roche LightCycler®, Stratagene Mx QPCR)
- Reaction tubes or cuvettes (and caps) for the appropriate real-time PCR thermocycler instrument
- Micropipettes and sterile pipette tips with aerosol barriers
- 1x TE (10mM Tris-HCl, pH8.0; 1mM EDTA), for "no template control"
- Microcentrifuge

Storage and Stability

The mastermix should be stored between -15°C and -25°C and is stable until the expiration date stated on the label. Avoid repeated (>2x) thawing and freezing of the mastermix. When working with the mastermix, keep it cold (on ice or cooling block) at all times, work quickly, and return any residual mastermix to freezer immediately after use. The positive control should be stored between -15°C and -25°C for long-term storage; working stocks of positive control DNA should be stored between 2° and 8°C for up to one month. Avoid repeated thawing and freezing of positive control DNA (>2x) as this may lead to degradation of the DNA. If the positive control is used only intermittently, it should be frozen in aliquots; each aliquot should be discarded after two freeze/thaw cycles.

Precautions

Real-time PCR is an extremely sensitive means for amplifying and detecting small quantities of DNA. Due care should be utilized to prevent any carryover contamination from previous PCR amplifications coming in contact with mastermix in this kit.

Recommendations to prevent carryover contamination:

- Store and extract positive or suspected positive material (such as test specimens, positive control, or previously amplified material) separately from all PCR kit components, e.g., in separate rooms.
- Aliquot mastermix into PCR reaction tubes or wells in the same BSL-2 cabinet in a DNA-free room.
- Add extracted DNA and positive control DNA to reaction tubes or wells in a room separate from the DNA-free room used for mastermix assembly.
- Use sterile pipette tips with aerosol barriers to avoid potential sample- to-sample contamination.
- Periodically (at least weekly), disinfect all work areas and pipettes with either a dilute bleach solution (i.e., 10% bleach) or ELIMINase® (Decon Labs). If using bleach, the 10% bleach solution should be made up fresh each week. Rinse surfaces with DNase/RNase-free water after decontaminating surfaces.

Thaw mastermix completely and mix all components by vortex and centrifuge briefly prior to pipetting into PCR reaction tubes, wells, or capillaries.

Limitations

This test should be used for testing DNA extracted from bovine fecal samples or for confirmation of cultures derived from bovine samples. No claims are made for other samples or for organisms derived from other animal species.

Specimen Collection and Storage

Fresh, moist fecal samples should be collected. Upon receipt in the testing laboratory, the fecal samples should be processed immediately; refrigerated (2°C to 8°C) for up to 1 day and then processed; or aliquoted, frozen and stored at -80^{O}C. Repeated freezing and thawing of fecal samples should be avoided, as DNA may degrade. Dry fecal samples can produce sub-optimal results and should be avoided.

Extraction of DNA from Fecal Sample from Culture Media

DNA should be extracted from fecal specimens or culture material using the MAP Extraction System (Tetracore, Cat. No. TC-9014-100). The use of other DNA extraction protocols or commercial products should be validated by the user.

Positive and Negative Controls

Positive Control:

A positive control is included in the kit and must be included with each test run. The positive control is a non-infectious synthetic template comprising a portion of the target gene sequence of the hspX gene of *M. avium ssp. paratuberculosis*. The positive control is supplied as a liquid solution containing 25,000 gene copies/2.5µl.

Negative control:

Negative (i.e., no template) controls must be included with each test run. A negative control is not included in the kit. However, the user is advised to use 1xTE as a no-template control (NTC).

A minimum of one positive and one negative control should be included in each test run.

Real-Time PCR Test Procedure

1. Determine the number of reactions needed.

   Remove the appropriate number of mastermix vials from the freezer and thaw. After the mastermix has thawed, vortex, and then briefly spin the vial(s) in a microcentrifuge before continuing.

2. Add 22.5µL of the mastermix to each thermocycler reaction tube or well.

   [NOTE: For LightCycler (Roche) only: add 17.5 µL of the mixture to LightCycler capillaries.]

3. Add 2.5µL of each extracted sample, no template control, and positive control to the appropriate reaction tube or well.

4. Close or cover each reaction tube or plate.

5. Briefly centrifuge reaction vessel.

   If using LightCycler® (Roche) capillaries, spin in a microcentrifuge to force the mix to the bottom of the capillary.

   If using the ABI 7500, iCycler (Bio-Rad), or Mx QPCR (Stratagene) instruments, briefly spin each plate to force the mix to the bottom of the well.

6. Observe the tube or bottom of the plate to ensure that there is no trapped air, which can interfere with the reaction.

7. Load the tubes, plates, or capillaries in the appropriate thermocycling instrument.

8. After an initial enzyme activation step at 95˚C, a two-step (95˚C, 62˚C) cycling reaction should be used. Cycling conditions on specific thermocycling instruments are given below (see **Cycling Conditions**).

Cycling Conditions

(A) ABI 7500 and BioRad iCycler iQ®

Cycling Program:

1. Enzyme activation step: 10 minutes @ 95°C
2. Two step PCR: (95°C x 15 seconds, 62°C x 60 seconds) for 45 cycles.

ABI 7500 Settings

- Set the quencher to "NONE".
- Select the reference dye as "ROX".
- Select "FAM" as dye layer.
- Reaction volume should be set to 25µL.

(B) Roche LightCycler®

Cycling Program:

1. Enzyme activation step: 10 seconds @ 95°C
2. Two step PCR: (95°C x 5 seconds, 62°C x 30 seconds) for 50 cycles.

LightCycler® Settings

- Initial denaturation step should set analysis mode to "NONE"
- Acquisition Mode = NONE
- For the 2-step cycling, analysis mode should be set to "Quantification"
- For the 95^{O}C step, Acquisition Mode = NONE
- For the 62^{O}C step, Acquisition Mode = SINGLE

(C) Stratagene Mx QPCR

Cycling Program:

1. Enzyme activation step: 10 minutes @ 95°C
2. Two step PCR: (95°C x 15 seconds, 62°C x 60 seconds) for 50 cycles.

Stratagene Settings

- Set the reference dye to ROX.
- Set FAM as the dye layer.

Interpretations of Test Results

The test is a fluorogenic probe hydrolysis assay that produces a characteristic fluorescent signal with a positive test. A positive sample is visualized as a sigmoidal curve on a two- dimensional linear grid where the X-axis represents the PCR cycle number and the Y-axis represents the relative fluorescence of the signal (see representative positive signals in Figure 1). The threshold cycle, or Ct value, is the cycle number at which the increase in the fluorescent signal associated with exponential growth of PCR product is first detected. Ct represents the point at which the signal exceeds the detection threshold and is dependent on the starting template copy number, i.e., the lower the Ct value, the more DNA template was originally present in the test sample at the initiation of the PCR reaction.

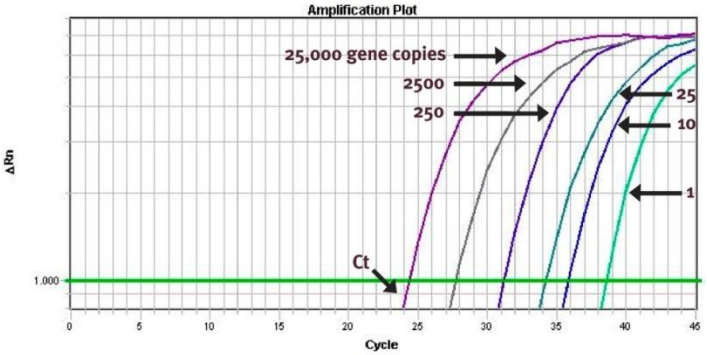


Figure 1. Amplification and detection of *M. avium ssp. paratuberculosis* DNA positive control on ABI PRISM® 7900; positive control was diluted from 25,000 gene copies to 1 gene copy.

A run is considered valid if:

(a) The amplification curve of the positive control provided (25,000 gene copies /2.5 µL) must have a cycle threshold value (Ct) that is between 20-26 cycles.

(b) The no-template control (NTC) must not cross the threshold prior to the endpoint of the test.

Ct cutoff for positive samples:

A sample is considered positive if:

- Ct $\leq$ 38 for ABI and BioRad series of real-time PCR thermocyclers
- Ct $\leq$ 42 for Roche and Stratagene real-time PCR thermocyclers

The Ct for a positive result has been set at the upper range of the Ct values at which one gene copy of DNA can be detected (in an uninhibited sample).

A sample that appears to cross the threshold after the cutoff Ct value cannot definitely be considered positive ("suspect" result). Such a sample may represent either a true negative or a low positive which was inefficiently amplified. To confirm such a suspect sample, the test should be repeated in duplicate, taking care to minimize any potential contamination. If at least one out of the two re-tested replicates is positive at an equal or earlier Ct value, the suspect sample can be classified as a "low positive." Low positive samples should be confirmed by culture.

NOTE: Caution should be used in interpreting positive samples at or below the cutoff value, since such results might be the result of transient passage of consumed organisms rather than a low-level infection.

Validation Studies

Fresh Bovine Fecal Samples

One hundred ninety-eight (198) fresh bovine fecal samples were evaluated in a blind trial with the Mycobacterium avium subspecies Paratuberculosis DNA Test Kit, Polymerase Chain Reaction (trade name: VetAlert™ Johne's Real-Time PCR from Tetracore, Inc.) and compared to results obtained by culturing the fecal samples on solid Herrold's Egg Yolk Medium (HEYM) for 16 weeks, a current gold-standard for detection of *M. avium ssp. paratuberculosis* in bovine fecal samples. The fecal samples were collected from dairy herds located in Pennsylvania, Maryland, Vermont, New York and California. All herds were known to contain a mixture of infected and non-infected cows. The DNA test kit correctly identified 75/75 culture positive samples containing $\geq$ 28 cfu/gram and also correctly

identified 88/88 culture negative samples. In addition, 35 samples produced low positive results (i.e., < 28 cfu/gram for culture or Ct > 33 for DNA Test) with either the DNA Test kit or culture, but not both. For these 35 samples, 5/35 were culture positive / DNA Test negative and 30/35 were culture negative / DNA Test positive.

In addition, a subset of seventy-seven (77) samples from the above study was cultured by serial dilution to obtain highly accurate estimates of bacterial counts, resulting in values between 0 and 3.6 million cfu/gram. Plotting the Ct value of the DNA Test against the log of the cfu/gram gave a correlation of $R^2 = 0.923$ (Figure 2).

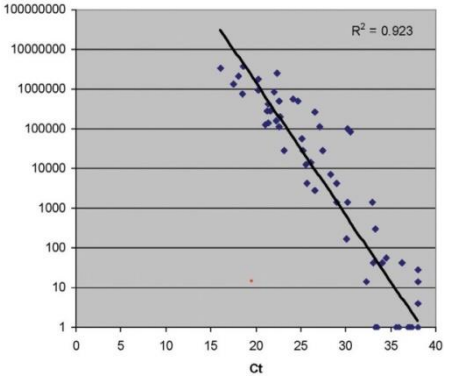


Figure 2. Correlation between the average Ct value of the DNA Test result and the log of the cfu/g (n=77).

Frozen Fecal Samples

Frozen archived fecal samples from 221 cows were evaluated in a blind trial comparing test results from the Mycobacterium avium subspecies Paratuberculosis DNA Test Kit, Polymerase Chain Reaction test with culture on HEYM (16 weeks). The fecal samples included 100 samples from non- infected cows (test negative, level 4 herd) and 121 culture positive samples of varying intensities. All samples (100/100) from the non-infected cows correctly gave a negative result in the test. The kit gave a positive test result in 59/60 cases when samples had an average cfu/tube count of greater than 3.0. When samples contained less than 3.0 cfu/tube, the kit gave a positive test result in 45/61 samples.

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VLN/PCN 644/5066.80

The use of the hspX gene for diagnostic detection of *M. avium ssp. paratuberculosis is* covered by US Patent No. 5,985,576, under exclusive license to Tetracore, Inc. from the USDA.

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