Validation and standardization of IS900 and F57 real-time quantitative PCR assays for the specific detection and quantification of Mycobacterium avium subsp. paratuberculosis.

Validation and standardisation of IS900 and F57 real time qPCR assays for the specific detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis.*

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Abstract

Mycobacterium avium subspecies *paratuberculosis* (Map) is the causative agent of Johneôs disease (JD), and may contribute to the onset and development of Crohnôs disease (CD) in humans. Due to its reported isolation from pasteurised milk and the potential for transmission of Map through environmental sources, rapid detection is fundamental. In this study, we developed two independent real time quantitative PCR (qPCR) assays targeting IS900 genetic insertion sequence and F57 sequence which proved able to detect and quantify Map DNA. Validation and standardisation of the developed methods were performed by evaluating diagnostic trueness, precision and accuracy of the techniques. Specificity of the IS900 and F57 methods was verified both *in silico* and experimental studies. Assays resulted very accurate and precise with good high repeatability and reproducibility. Moreover, the two real time assays were very specific for Map, discriminating most of mycobacterial and non-mycobacterial species.

Key words: Real time qPCR assay, *Mycobacterium avium* subspecies *paratuberculosis*, IS900 and F57 sequences.

Introduction

Mycobacterium avium subspecies *paratuberculosis* (Map) is an acid-fast mycobactin dependent pathogen that causes a chronic progressive granulomatous enteritis known as paratuberculosis or Johneôs disease (JD) (Whittington and Sergeant 2001). Its primary hosts are domestic and wild ruminants, including cattle, sheep and goats (Bauerfeind et al. 1996). It is estimated that about 40% of United States herds are infected with Map, resulting in considerable economic losses to the dairy industry totalling more than $200 million *per annum*.

Clinical signs similar to those of paratuberculosis in ruminants are characteristic **for** Crohnôs disease (CD) in humans. CD is a chronic inflammation of distal intestines exhibiting a pathology similar to that of JD in ruminants. This has led to the hypothesis that Map could play a role in the development of CD (Ayele et al. 2001; **Feller et al. 2007**; Skovgaard 2007; Uzoigwe et al. 2007; Behr and Kapur 2008). The prevalence of CD is estimated to be 0.15% among the United States population resulting in substantial morbidity and medical costs (Ashford et al. 2001). Due to its reported isolation from pasteurised milk and the potential for transmission of Map through environmental sources, rapid detection is fundamental. A number of conventional PCR assays specific for Map detection have been described (Grant et al. 2000; Corti and Stephan 2002; Pillai and Jayarao 2002; OôMahony and Hill 2004). Most of these PCR methods target the insertion sequence 900 (IS900), a species-specific insertion element in Map, which has mostly been accepted as a standard marker (Green et al. 1989; Vary et al. 1990; De Lisle et al. 1992; McFadden et al. 1992; Bauerfeind et al. 1996; Millar et al., 1996; Portillo et al. 1996; Stevenson and Sharp 1997; Secott et al. 1999; Bull et al. 2000; Coetsier et al. 2000; Grant et al. 2000; Marsh et al. 2000; Corti and Stephan 2002; Pillai and Jayarao 2002). However, several publications report the presence of IS900-like sequences in other closely related environmental mycobacterial species, which could negatively affect the specificity of PCR assays (Cousins et al. 1999; Englund et al. 2002). In the past few years, other Map-specific genetic elements have been described at low copy numbers.

These elements include mainly the F57 sequences (Poupart et al. 1993; Tasara and Stephan 2005; Herthnek and Bölske 2006). These sequences have been found only in Map making them a potentially specific target and, although F57 sequences may not be as sensitive as the multicopy IS900 elements, they are highly specific for Map making them less prone to false-positive results (Poupart et al. 1993; Coetsier et al. 2000; Vansnick et al. 2004; Tasara et al. 2005).

In this study, we have developed two specific and sensitive real time quantitative PCR (qPCR) assays targeting IS900 genetic insertion sequence and F57 sequence for detection and quantification of Map DNA.

2. Materials and methods

2.1. Bacterial and viral isolates

Prototype *M. avium* subsp. *paratuberculosis* (Map) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). *M. avium* subsp. *paratuberculosis* strain ôLindaô (ATCC 43015), originally isolated from a patient with CD, was incubated at 37°C for 10 weeks in the ATCC Medium prepared by adding to Middlebrook 7H9 Broth (Difco Laboratories, Detroit, Mich.): agar technical (14 g/l; SIFIN, Berlin, Germany), tween 80 (0.5 g/l; Sigma Chemical Co., St. Louis, Mo.), mycobactin J (2 mg/l; Allied Monitor, Fayette, MO, USA) and Dubos Oleic Albumin Complex (100 ml/l; Difco Laboratories), as described by ATCC Product Information Sheet. Single colonies were observed after four weeks. To evaluate the specificity of the Map real time qPCR assays which we developed, purified DNA templates from eight Map isolates, five non-Map *Mycobacterium* spp. isolates, and 10 non-*Mycobacterium* spp. and viral isolates were used for inclusivity and exclusivity testing (Table 1A and 1B).

2.2. DNA extraction

After the growth of Map colonies, nucleic acid of a single Map colony was isolated by the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the modified protocol ñPurification of total DNA from animal blood or cells (Spin-Column Protocol)ò, listed in the manufacturerôs handbook. As a modification, a mechanical lysis step was included in the original protocol. In particular, lysis efficiency was improved by colony disruption using the rotor-stator homogenizer TissueRuptor (Qiagen). Briefly, **colonies were** harvested into 180 µl of buffer ATL **(Tissue Lysis Buffer, Qiagen)** and homogenized using the rotor-stator homogenizer TissueRuptor (Qiagen) for 30 s to obtain a homogeneous mixture. Twenty microliters of proteinase K (2mg/ml) were added, followed by vigorous vortex. After incubation at 56°C for 60 min, 200 µl of buffer AL

and 200 µl of ethanol were added; the combination was vortex mixed thoroughly. Subsequently, the entire mixture was loaded on a DNeasy mini column and centrifuged for 1 min at 6,000 x g. The column was washed once with buffers AW1 and AW2, respectively, and DNA was eluted 2 times each with 200 µl buffer AE.

2.3. Primers and probes for IS900 and F57 real time qPCR assays

Specific real time qPCR primers for the IS900 and F57 fragment and probes were designed using Primer Express Software Version 3.0 (Applied Biosystems, Cheshire, United Kingdom). Probes were labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) on the 5ô end and with the quencher dye Nô, Nô, Nô, Nô-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3ô end. Primers and probes were aliquoted to a final concentration of 10 pmol/µl and stored at -20°C. The primers and probes used in this study are shown in Table 2.

2.4. IS900 and F57 real time qPCR assays

The conditions for the IS900 and F57 real time qPCR assays were optimised until the best primer and probe concentrations and cycling conditions were determined. Real time qPCR assays were performed using the Platinum qPCR SuperMix-UDG containing ROX as a passive reference (Invitrogen). IS900- and F57-specific forward primers, reverse primers and probes were used at the optimized concentration of 0.2 µM, 0.2 µM and 0.1 µM, respectively. Five microliters of DNA were added to 15 µl of the reaction mix, giving a final reaction concentration of 20 µl. Uracil-DNA glycosylase was used to eliminate PCR contamination from previous PCR reactions. Real time qPCR assays were performed using the 7500 Real Time PCR System (Applied Biosystems) instrument. Cycling conditions to optimize amplification profile included an initial decontamination at 50°C for 2 min, denaturation step at 95°C for 10 min in order to activate DNA polymerase and an amplification that was performed during 45 cycles including denaturation (95°C for 15 s), annealing and extension (60°C for 1 min). The cycle number during which the fluorescence signal is above

the background (Ct) is proportional to the initial log concentration of the target DNA. Analysis of the real time qPCR assays was performed using the RTS Analysis Software 2.0 (Applied Biosystems).

2.5. Specificity of real time qPCR assays

The specificity of the developed real time qPCR assays was verified by *in silico* studies (analytical specificity) against publicly available sequence databases (BLAST alignment software (www.ncbi.nlm.nih.gov/blast/) to evaluate possible cross-reactions with non-mycobacterial species and viral isolates. Experimental specificity was also verified. In particular, non-*Mycobacterium* spp. and viral sequences of different isolates were used for exclusivity testing (Table 1B). Cross-reactions with human DNA sequences were excluded by testing the primers against preparations of human nucleic acids.

2.6. Preparation of quantification standards for sensitivity of qPCR assays

Map purified DNA concentration was spectrophotometrically estimated at OD_{260} by using a high-resolution spectrometer. The Map DNA concentration value was 93.8 ng/µl. The exact Map genome copy number was calculated from the molecular weight of Map DNA (3,187,655,460 MW) and Avogadro number (6.023×10^{23}) to obtain stock preparations containing 10^7 genomic DNA copies in one microliter. Stock preparations at 2×10^6 copies/εl were diluted to 2×10^{-1} copies/εl by a series of 10-fold dilutions. To determine the sensitivity of the PCR assays, we have used genomic DNA dilutions (from 2×10^6 copies/εl to 2×10^{-1} copies/εl) to estimate the dynamic range. Efficiency, defined as the rate of amplification that leads to a theoretical slope of 3.32 with an efficiency of 100% in each cycle, was also evaluated. Efficiency can be calculated by the following equation: $10^{(-1/slope)}$ (Rasmussen et al. 2001). For storage, dilutions of the standards were frozen in aliquots, and when needed, the aliquots were thawed before use and than stored at 4C° during use.

2.7. Validation and standardisation of IS900 and F57 real time qPCR assays

To determine the performance of IS900 and F57 assays, we assessed the diagnostic trueness, precision and accuracy of the techniques. In particular, precision was assessed by evaluating repeatability and intermediate reproducibility of IS900 and F57 assays. To determine the repeatability, several replicates containing the various amounts of Map DNA were tested. The repeatability was determined by 10-fold serial dilutions of the IS900 and F57 quantification standards. In particular, we used four different dilutions (10^2, 10^3, 10^4, 10^5 copies/reaction) of quantification standards. Each dilution was analysed ten times, with the same method on identical test items in the same laboratory by the same operator using the same equipment. As concern intermediate reproducibility, each dilution was analysed with the same method on identical test items in ten different runs performed by three different operators using different equipment on different days. Moreover, we used the Dixonôs test to examine if one measure from ten replicate measures that we performed (10^2, 10^3, 10^4, 10^5), could be rejected or not and the Shapiro-Wilkôs test to compare these measures against the Normal distribution. Statistical data analysis were performed using the PASW Statistics 18.0 (SPSS Statistics) software.

2.8. Clinical specimens

To verify whether the developed assays could be of practical use, a total of 10 colonic mucosal biopsy specimens were analysed by IS900 and F57 real time qPCR assays. In particular, specimens consisted of 5 colonic biopsies from patients with CD, and 5 biopsy samples from patients with colon carcinoma (control group). **The case definition of CD was established on the basis of standard clinical, radiological, endoscopic, and histopathological criteria. The CD group consisted of 5 Italian patients (4 males, 1 female; median age, 36 years, range, 28-43) with endoscopically active inflammatory disease. None of the patients were receiving anti-Map**

treatment. The control group comprised 5 Italian patients (2 males, 3 females; median age, 57 years, range, 47-70) with colon carcinoma who did not have a clinicopathological diagnosis of CD; only specimens of normal colon outside the area with cancer or dysplastic lesions were considered. None of the individuals in the control group was receiving antibiotics. A number of precautions were undertaken to prevent the occurrence of false-positive results. Each run included control reactions lacking template (no-template controls) to test for the presence of contamination or the generation of non-specific amplification products under the assay conditions used. The presence of cellular GAPDH gene was analysed as internal control and marker of sensitivity of the assays.

3. Results

3.1. Sensitivity of IS900 and F57 real time qPCR assays

Optimal real time qPCR assay conditions that allowed efficient amplification of the IS900 and F57 target sequence were established. In particular, efficiency and sensitivity of IS900 and F57 real time qPCR assays were assessed by repeated testing of serial logarithmic dilutions of the quantification standards copies. After real time qPCR amplification, the Ct value (crossing point of the amplification curve with the pre-set threshold of fluorescence detection) of individual dilution steps was plotted against the initial bacterium copy number, leading to a typical standard curve. To examine the dynamic range (range of concentrations over which the method performs in a linear manner with an acceptable level of trueness and precision) of IS900 and F57 genes quantification by real time qPCR, serial dilutions of the quantification standard ranging from 10^7 to 1 copies/reaction were carried out. ~~**As shown in Fig. 1,**~~ IS900 real time qPCR assay was able to quantify from 10^7 to 1 copies/reaction, whereas F57 real time qPCR assay was able to quantify from 10^7 to 10 copies/reaction. The consistency of replicates was measured by the correlation coefficient (R^2), which indicates the linearity of Ct values plotted in the standard curves. The R^2 index for IS900 and F57 genes was 0.999 and 0.996, respectively and the standard curve slope was 3.461 and 3.383, respectively. Sensitivity of real time qPCR assays was determined by the lowest standard dilution consistently detectable in replicate reactions at frequency of 100%. IS900 and F57 sensitivity was 1 copy/reaction and 10 copies/reaction, respectively. Efficiency reflects the quality of the real time qPCR and can be calculated from a standard curve generated using the concentration of the quantification standard serial dilutions. The efficiency of the reaction is considered acceptable if it falls between the range of 1.7 and 2.2, with 2 being ideal. The efficiency of our two real time qPCR assays was 1.9 for IS900 and 2 for F57.

3.2. Validation and standardisation of IS900 and F57 real time qPCR assays

Diagnostic trueness of IS900 and F57 real time qPCR methods, defined as the degree of agreement between the average value obtained from a large series of test results and an accepted reference value, was evaluated. To establish the level of trueness and concordance with the assigned value, data from ten replicate measures of each dilution that we performed (10^2, 10^3, 10^4, 10^5) were analyzed using a Studentôs *t*-test to compare the mean concentrations from each dilution to an accepted reference value. The mean concentrations from each dilution for the two methods, are shown in Table 3 with the *t*-test results, which indicate the significance of the differences between each experimental mean and the assigned value. Analysis of the *t*-statistics showed that both methods had *t*-calc values lower than the *t*-tab value, demonstrating a significant trueness of IS900 and F56 assays.

Precision of methods was expressed as the coefficient of variation (CV) in the log10 values of the concentration. Repeatability and intermediate reproducibility of IS900 and F57 assays were evaluated over different concentrations ranging from 10^2 to 10^5 copies/reaction from ten replicate measures (n=10) of each reference bacterial quantification standard within a single run or in ten different run experiments performed by three different operators. The precision associated with each dilution measurement (10^2, 10^3, 10^4, 10^5) was assessed by calculation of the CV for each. The coefficients of variation within a single run (repeatability) ranged from 2,99% 18,57% whereas the coefficients of variation in different runs (intermediate reproducibility) ranged from 4,8% to 40,1% (Table 3).

Diagnostic accuracy includes both, trueness and precision. The measure of accuracy is usually expressed numerically in terms of bias (lack of agreement). Accuracy shall be within ±25% of the accepted reference value over the whole dynamic range. Data for the percentage of inaccuracy IS900 and F57 methods are reported in Table 3.

3.3. Specificity of real time qPCR assays

The ónucleotide-nucleotideô search for nucleotide sequences performed at the National Center for Biotechnology Information and the National Library of Medicine web site confirmed that the primer pairs used amplify the following Map sequences: (accession numbers GQ144322.1, FJ775182.1, FJ775181.1, EU714038.1, EU057175.1, EU057174.1, EU057173.1, EU057172.1, EU057171.1, EU057170.1, EU057169.1, EU057168.1, EU057167.1, EU057166.1, EU057165.1, EU057164.1, EU057158.1, EU057157.1, EU057156.1, EU057155.1, EU057153.1, EU232753.1, EU232752.1, EU232748.1, EU232747.1, EU130943.1, EF514833.1, EF514831.1, EF514829.1, EF514828.1, EF514825.1, EF514824.1, EF514818.1, EF536058.1, EF536056.1, EF536055.1, EF536048.1, EF536047.1, EF536046.1, EF536044.1, EF536043.1, EF536042.1, EF536041.1, EF536040.1, EF536039.1, EF536038.1, EF015397.1, S74401.1, AF416985.1, AF305073.1, AJ250018.1, AJ250015.1, AJ011838.1, AY974348.1, AY974347.1, AE016958.1, AB052552.1, AJ250023.1, AJ251437.1, AJ251436.1, AJ251435.1, AJ251434.1, AJ250022.1, AJ250021.1, AJ250020.1, AJ250019.1, AJ250017.1, AJ250016.1, X16293.1, AY974346.1, AY974345.1, EU714041.1, EU714039.1, EU714037.1, EU714035.1, AF455252.1, as concerns IS900 sequences and GQ140314.1, EU379657.1, AE016958.1, X70277.1, as concerns F57 sequences) and do not amplify other bacteria pathogenic to humans. Moreover, IS900 and F57 primer and probe sets, tested on Map isolates, were able to detect only their respective subspecies genomes, thus being the inclusivity of 100% (Table 1A). The assayôs specificity was further demonstrated by its ability to exclude all non-*M. avium* subsp. *paratuberculosis* bacterial species and viral isolates listed in Table 1. No positive results were demonstrated for the other bacterial and viral isolates indicating that these molecular assays are highly specific for Map isolates, thus being the exclusivity of 100% (Table 1B).

3.4. Detection of Map-DNA in clinical specimens

Map-DNA was detected separately and concordantly in 60% (3/5) of patients with CD and in 0% (0/5) of control group by both IS900 and F57 real time qPCR assays. Therefore, the two developed methods were in agreement. All negative control reactions were PCR negative, demonstrating the absence of amplicon contamination. Positive samples were confirmed by sequencing.

4. Discussion

Map represents an important pathogen for the dairy industry around the world. The difficulties involved in detecting and enumerating this organism are well known. ~~Currently, there is no reliable approach of determining the total number of Map cells. In fact, conventional plating of Map cells and the counting of colonies is not accurate because the Map cells forms clusters and the number colonies does not reflect the real number of cells.~~ Traditional diagnostic methods include culture of clinical samples (especially feces and tissue) and serologic tests. Although culture is considered the gold standard, this method is fraught with difficulties. Some of the major difficulties of working with Map are its slow growing nature and fastidious nutritional requirements. The microorganism takes at least 12 to 16 weeks to grow to detectable levels, and even the most sensitive culture methods have only 50% sensitivity. In addition, the chemical decontamination steps, used before cultivation to prevent culture overgrowth by competing microflora present in samples, could inactivate Map, with further reduction of its diagnostic value (Chiodini et al. 1984; Stabel et al. 2002; Feller et al. 2007). Immunological-based detection methods are faster than culture methods but are hampered by low sensitivity and cross-reactivity problems (Ferreira et al. 2002). In particular, serologic tests, such as enzyme-linked immunosorbent assays (ELISA), complement fixation (CF) and agar gel immunodiffusion (AGID), are limited in their use because of low specificity and sensitivity (Nielsen et al. 2000; Kalis et al. 2002; Stabel et al. 2002). Seroconversion, in fact, occurs relatively late during the course of the disease. In particular, ELISA can be performed in few hours, but their sensitivity is estimated at 45% since antibodies may not be detectable until late in infection (Nielsen et al. 2002). Therefore, sensitive and specific PCR assays for detection of Map could contribute immensely to research efforts aimed at understanding **the potential role of this organism in human CD** and its transmission by milk product. Rapid real time assays are becoming increasingly popular where fast and accurate diagnosis of Map is required. Currently, several Map detection PCR methods are widely available,

but practical limitations remain. First of all, detection of Map isolates has been based mainly on IS900 PCR methods (Millar et al. 1996; Corti and Stephan 2002; Kim et al. 2002; Pillai and Jayarao 2002; Rodrìguez-Làzaro et al. 2005; Herthnek and Bölske 2006). IS900 is defined as a 1,451-bp multicopy element inserted into 14 to 18 conserved loci in the Map genome and different studies suggested that it was exclusively present in Map (Green et al. 1989). Hence, IS900 has been the marker of choice for most molecular assays. However, as IS900-like sequences have been demonstrated in other unrelated *Mycobacterium* species, it is evident that the PCR systems used for IS900 are not completely specific for Map. This evidence has resulted in doubts concerning the current Map detection methods (Roiz et al. 1995; Cousins et al. 1999). It is therefore desirable to use alternative IS900 PCR systems to confirm a positive PCR for Map. Therefore, because of the importance of distinguishing these related mycobacteria, there is a need of adding other molecular targets to IS900 sequence to confirm the presence of Map. In the present study we successfully developed and optimised two independent real time quantitative PCR (qPCR) assays able to detect both IS900 and F57 elements. In contrast to IS900, F57 has no known similarities to genes on other related organisms, which made the task of selecting suitable oligos for F57 less complicated. In particular, F57 sequence is a genetic element that is currently known to exist only in Map and that so far has been found to be highly specific. Therefore, the identification of this DNA sequence considered to be unique to Map, offers additional tools for rapid identification of this organism.

Plus, previous works have shown that Map is very difficult to detect reliably and reproducibly by PCR methods (Naser et al. 1999). In particular, different critical steps in the DNA extraction procedure were found to be of importance. In fact, Map cells are structurally complex and must be efficiently lysed to release as much of the target DNA molecules as possible. We used a DNA extraction procedure that was rapid and able to obtain a high-quality Map DNA. This protocol was a modified protocol that combined mechanical lysis and subsequent template purification using nucleic acid binding columns. By integrating the optimised Map DNA extraction procedure and optimised IS900 and F57-based primer and probe concentrations and cycling conditions, we

obtained two independent real time quantitative PCR assays very sensitive and specific able to detect DNA Map. In particular, the sensitivity of the IS900 assay was shown to range from 1 to 10^7 copies/reaction, whereas F57 real time qPCR assay was able to quantify from 10 to 10^7 copies/reaction.

Diagnostic trueness, precision and accuracy of the developed methods was also evaluated. Both IS900 and F57 assays resulted very accurate and precise with good high repeatability and reproducibility as reflected by the CV results (Table 3). Moreover, the two real time assays that we developed were very specific for Map, discriminating most of mycobacterial species and non-mycobacterial species employed in this study.

In summary, we have presented two rapid, sensitive and specific real time qPCR assays that can be used to quantify DNA Map. The IS900 and F57 real time qPCR assays that we developed could be useful to enlarge the spectrum of available Map detection methods and contribute to the routine detection of this organism as valuable tools in the molecular diagnostics of Map. In conclusion, after validation on several mycobacterial strains and clinical samples, our molecular assays were found to be both sensitive and reliable.

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Table 1. Evaluation of real time PCR specificity with *Mycobacterium avium* subsp. *paratuberculosis* and non-*Mycobacterium avium* subsp. *paratuberculosis* strains and viral isolates. Inclusivity testing (A); exclusivity testing (B).

Taxon	Source	Test specificity for:	
		IS900	**F57**
M. avium subsp. *paratuberculosis* strain Linda (human)	ATCC 43015	+	+
M. avium subsp. *paratuberculosis* strain 5617 (bovine)	ATCC 19698	+	+
M. avium subsp. *paratuberculosis* strain NCTC 8578 (cow)	ATCC 19851	+	+
M. avium subsp. *paratuberculosis* strain Ben (human)	ATCC 43544	+	+
M. avium subsp. *paratuberculosis* strain Dominic (human)	ATCC 43545	+	+
M. avium subsp. *paratuberculosis* strain Holland-1 (human)	ATCC 49164	+	+
M. avium subsp. *paratuberculosis* strain 97R0816 (cow)	ATCC 700535	+	+
M. avium subsp. *paratuberculosis* strain K10 (bovine)	ATCC BAA-968	+	+

Table 1A.

Taxon	Source	Test specificity for:	
		IS900	**F57**
Mycobacterium bovis	ATCC 27289	-	-
Mycobacterium microti	ATCC 11152	-	-
Mycobacterium tuberculosis	ATCC 25177	-	-
Mycobacterium africanum	ATCC 25420	-	-
Mycobacterium caprae	ATCC BAA-824	-	-
E. coli	ATCC 11605	-	-
Streptococcus agalactiae	ATCC 13813	-	-
Staphylococcus aureus	ATCC 10832	-	-
Enterobacter cloacae	ATCC 10699	-	-
Bacillus cereus	ATCC 10987	-	-
Salmonella enterica	ATCC 10398	-	-
Virus		-	-
Coxsackievirus	ATCC VR-1005PI/MK	-	-
Echovirus	ATCC VR-1038	-	-
Enterovirus	ATCC VR-1077	-	-
Adenovirus	ATCC VR-1086	-	-

Table 1B.

Table 2. PCR primer and probe sequences to amplify the multicopy element IS900 and the single copy element F57 of *Mycobacterium avium* subsp. *paratuberculosis.*

Target gene	Primer/probe	Sequence (5ĉ-3ô)	Product (bp)
IS900	IS900QF	CCGGTAAGGCCGACCATTA	67
ATCC no.	IS900QR	ACCCGCTGCGAGAGCA	
19698	IS900QP	FAM-CATGGTTATTAACGACGACGCGCAGC-TAMRA	
F57	F57QF	AACTAAGCGGATCGACAATTC	80
Accession no.	F57QR	TGGTGTACCGAATGTTGTTG	
X70277	F57QP	FAM-TGCAACTCGAACACACCTGGGA-TAMRA	

Table 3. Statistical summary of validation and standardisation of IS900 and F57 real time qPCR assays.

	10^2	10^3	10^4	10^5
IS900-Trueness (t-test):				
experimental mean concentration	108,9701215	1057,173703	10976,67223	109851,658
standard deviation	8,935402709	107,3067791	1521,132919	8549,866926
t-calc	2,2447563	1,191390579	1,435709845	2,576528626
t-tab (n=10)	2,776	2,776	2,776	2,776
F57-Trueness (t-test):				
experimental mean concentration	83,00521733	900,4376291	9942,589826	100414,7937
standard deviation	15,41613442	93,53565816	1110,845071	3006,841907
t-calc	2,465046573	2,380142863	0,115563417	0,30846549
t-tab (n=10)	2,776	2,776	2,776	2,776
IS900-Precision (% coefficient of variation, CV):				
repeatability	8,199864865	10,1503451	13,85786955	7,783102308
intermediate reproducibility	21,05633093	17,19892394	25,92358547	10,8523951
F57-Precision (% coefficient of variation, CV):				
repeatability	18,57248847	10,38779979	11,17259276	2,994421236
intermediate reproducibility	40,13820884	15,26099457	21,70100988	4,805707451
IS900-Accuracy (% bias inaccuracy):	8,970121537	5,717370267	9,766722338	9,851657958
F57-Accuracy (% bias inaccuracy):	16,99478267	9,956237086	0,574101744	0,414793723