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"Development of a molecular diagnostic test applied to experimental abattoir surveillance on bovine tuberculosis".

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Abstract

One of the most essential systems applied to the eradication of bovine tuberculosis by *Mycobacterium bovis* is the epidemiologic surveillance of animals slaughtered in abattoir by means of inspection and sample taking of lesions compatible with tuberculosis, confirming the existence of the disease through culture and molecular detection, which takes weeks before a result can be obtained. An interesting alternative is to develop high-throughput molecular systems for the direct detection of *M. bovis* on biological samples. In this sense, our research has developed a molecular detection system by means of a real time based PCR process which is applied directly to bovine biological samples and it allows to differentiate between *Mycobacterium tuberculosis* Complex, *Mycobacterium avium* Complex and other atypical mycobacteria that are interesting from the veterinary point of view. The sensitivity was analyzed by applying a conventional extraction system based on guanidine thiocyanate and a robotized system based on the selective magnetic capture of mycobacterial DNA. The molecular detection system showed a high specificity and a detection threshold of only 2-3 genomes. The sensitivity depended on the DNA extraction system being used and on the kind of lesions on which it was used; the sensitivity ranged from 61.11% for samples with non visible lesions to 80.64% for chronic lesions, with an average sensitivity of 73.87% when using the manual extraction system and between 27.77 and 74.19% (average sensitivity 47.74%) when using the automated robotic system. In conclusion, our multiplex real time PCR assay represents a fully controlled, high-throughput diagnostic tool for the rapid detection of *Myobacterium* presence directly in animal clinical specimens, which could be a practical tool in the context of bovine tuberculosis abattoir surveillance programs and granuloma submission programs.

Key words: bovine tuberculosis, molecular diagnostic, epidemiologic surveillance.

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Introduction

Bovine tuberculosis (caused by *Mycobacterium bovis*) is still an infectious disease that causes substantial damages in the agricultural sector in many developed countries; it is nowadays subjected to expensive eradication programmes in most EU countries, Spain among them (Reviriego Gordejo and Vermeersch, 2006). The development process of the eradication programmes usually shows a tendency to establish epidemiologic surveillance systems in abattoirs, where not only the sanitary inspection of all the animals slaughtered for consumption purposes is carried out but also, the systematic collection of granulomas or lesions which are compatible with tuberculosis for their subsequent culture, disease confirmation and epidemiological trace back of the outbreak, which usually ends in the total culling of the herd that have tested positive by means of the agent isolation (Evans, 1972; Kaneene et al., 2006; More and Good, 2006; Radunz, 2006). In Spain, the development of the eradication programme is allowing a progressive decreasing of the prevalence, (0.30% of prevalence in animals in the year 2005. Source: RASVE-MAPA http://rasve.mapa.es/) in such a way that it is advisable to start the abattoir epidemiological surveillance systems, which were initially developed in parallel with the field eradication system, by means of diagnosis and slaughtering, and they will be possibly the main tool for the eradication system in the future. Under these circumstances, it is necessary to develop diagnostic systems which are directly applied to biological samples and enable a high level of achievement and good levels of sensitivity and specificity, allowing a quick diagnosis in the laboratory by using the samples taken in the abattoir.

In this sense, it is worthwhile pointing out the molecular diagnostic systems, especially those ones based on the real time PCR technology (Q-PCR), due to the fact that they are faster and show more automation possibilities. So, although a lot of real time molecular detection systems, which are directly applied to biological samples, have been recently developed in order to carry out human tuberculosis diagnosis (caused by *M. tuberculosis*) (Broccolo et al., 2003; de Kantor and Portuondo, 1974; Desjardin et al., 1998; Eishi et al., 2002; Heginbothom et al., 2003; Kramme et al., 2003; Kraus et al., 2001; Lachnik et al., 2002; Miller et al., 2002; Shrestha et al., 2003). The development of procedures for *M. bovis* has been very limited and there has existed

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just one significant research made on bovine tissue samples (Taylor et al., 2001). The main difference between the systems developed for the human and bovine species is not based on the kind of genetic marker, but on the extraction system to be used, due to the difficulty in extracting mycobacterial DNA in bovine samples, as they have few bacteria and because of the structure of the biological sample itself, which shows strong fibrosis and calcification that hamper the access to the DNA to be detected (Liebana et al., 1995). The result is a limited sensitivity, which varies between 70-75% (Bollo et al., 1998; Liebana et al., 1995; Taylor et al., 2001) and is lower than that of the culture (Taylor et al., 2001). In order to solve this problem, mycobacterial DNA selective capture systems have been developed (SC) (Brugiere et al., 1997; Mangiapan et al., 1996; Roring et al., 1998; Roring et al., 2000; Taylor et al., 2001); these systems are liable to be automated in robotic platforms. In this way, as well as developing a molecular detection system, we also evaluate that procedure (SC) by comparing it to the conventional DNA extraction systems by means of organic solvents and denaturing agents.

2. Material and Methods

2.1 Bacterial strains

The mycobacterial strains used for sensitivity and specificity testing and for optimization of the real time PCR are listed in Table 1. The strains were Institute Pasteur Collection (CIP) strains. All mycobacterial strains were cultured in liquid Dubos medium. Additionally, 7 strains belonging to 7 different species were also used in specificity tests. Some of these species are established pathogens causing lymphadenitis, and other ones are normal inhabitants of the bovine oropharynx, as the species belonged to the genera *Actinobacillus, Actinomyces, Arcanobacterium, Bordetella, Corynebacterium, Haemophilus* and *Streptococcus*. The identification of the reference strains was carried out by molecular means (Cousins et al., 1991; Liebana et al., 1996).

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2.2. Biological material

Retropharyngeal and mediastinal lymph nodes were collected from 125 cattle. These cattle were either intradermal skin test positive and identified as having TB like-lesions (confirmed by Ziehl-Neelsen staining process and microscopic examination), during routine meat inspection, or without lesions (NVL). The samples were divided into similar portions, keeping one of them at -80ºC, in case the tests were to be repeated. The Hexadecylpyridinium Chloride method (Corner, 1988) was used as a decontamination procedure, showing in parallel and in duplicate two Lowenstein-Jensen test tubes with pyruvate and two Lowenstein-Jensen test tubes with glycerine (Pronadisa, Spain). Residual decontaminated tissue was stored at -20ºC for sequence capture. The identification of the *Mycobacterium tuberculosis complex,* isolated from culture, was carried out by molecular means (Cousins et al., 1991; Liebana et al., 1996). Each sample was identified in duplicate, including negative controls (just water) and positive ones (*M. bovis* CIP 105050).

2.3 DNA extractions from culture

A loopful, taken from a Lowenstein–Jensen pure culture, grown with pyruvate and positively identified as belonging to the *Mycobacterium tuberculosis* complex, was transferred to a microcentrifugal vial containing 100 ml 10 mM Tris–HCl/1 mM EDTA and incubated for 20 min at a temperature of 99ºC. After centrifugation, the supernatant was stored at -20ºC until used. Serial dilutions of *M. bovis* CIP 105050 genomic DNA (1 µg/ml to 1 femtograms/ml) were used as positive controls.

2.4. DNA extractions from bovine tissue

i. Sequence capture: DNA sequence capture PCR, as previously described (Taylor et al., 2001), was applied to homogenates of 125 decontaminated bovine tissues with the following additional modifications. In brief, suspensions of decontaminated tissue homogenate (500 µl) were transferred to a screw-cap microcentrifuge tube containing 500 µl of 0.1-mm-diameter glass beads (Cell Disruption Media, 0.1 mm. Scientific Industries Inc.). The pellet and beads were

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suspended in 500 µl of lysis buffer (0.01 M Tris-HCl (pH 7.8), 0.01 M EDTA and 0.5% SDS). Samples were agitated in a FastPrep 101 bead shaker at 6m/s for 1 min. Proteinase K (Takara Bio Inc. Shiga, Japan) was added to a final concentration of 20 mg/ml and the mixture was incubated at 55ºC over-night in a ThermoMix Confort (Eppendorf, Hamburg, Germany) under constant agitation at 550 r.p.m. The samples were shaken in the FastPrep as before. Following a brief centrifugation, the resulting supernatant was subjected to an automated protocol in an Evo Freedom 150 (Tecan, Maennedorf, Switzerland) liquid handling robot.

The EVO Freedom 150 was equipped with 8 disposable tip adapter, the Te-MagS™ instrument (Tecan, Maennedorf, Switzerland) (48 tubes of 1.5 ml format), carrier for primary 2 ml sample tubes, for reagents, for disposable tips and PCR microplates positioned on the work table. A robotic manipulator arm (RoMa) transports the microplates from the pipeting positions to the Te-MagS™ instrument. The magnetic separation module is controlled from the Tecan Gemini Software (Tecan, Maennedorf, Switzerland) by serial interface.

500 µl were taken from the supernatant of each sample and they were subjected to a 99º C denaturing process for 15 minutes using the Te-MgS^{TM} instrument. Afterwards, the capture biotinized oligonucleotides were added at room temperature (Ec16S.1390 y Mb23S.44n) (2.5 pm of each one) in 5 M of NaCl. The homogenate was shaken in the Te-MgS^{TM} , allowing in this way the hybridization of the capture probes for 2 hours at 65ºC under constant agitation.

After the hybridization process 10 µl of Streptavidin Magnetic Particles (Roche Diagnostics, Penzber, Germany), dissolved in binding buffer (Binding Buffer: 10 mM Tris-ClH, 1mM EDTA in 100 mM NaCl pH 7.5) and they were incubated for 3 hours, allowing in this way the instrument Te-MagS to carry out the movement of the particles inside the screw-cap tube. The mangnetic beads joined to the binding made up of the capture probe and the bacterial DNA were washed twice using the mangnetic separator of the Te-MagS in 750 µl of wash-buffer (Wash-buffer TEN1000: 10mM Tris-ClH, (pH 7.5) 1mM EDTA, 1M NaCl,) and then in sterile deionized H_2O.

Magnetic beads were resuspended in 25 ml of sterile deionized H_2O and stored at -20°C prior to PCR amplification. Immediately prior to amplification, captured mycobacterial DNA was

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released from magnetic beads by heat treatment at 99°C for 5 min. Following a brief centrifugation, the resulting supernatant was subjected to the appropriate amplification in the Real Time PCR system.

ii. Guanidinium thiocyanate DNA purification. For the extraction of DNA directly from bovine tissue biological samples, the reagent Tri-Reagent was used (Molecular Research Centre, Cincinnati, USA), based on the Guanidinium thiocyanate action, according to the instructions of the manufacturer and in the way described in other studies (Brisson-Noel et al., 1991; Chakravorty and Tyagi, 2001). This extraction was carried out in parallel, both on the same samples described before and on 500 µl of decontaminated tissue homogenate. On both kinds of extraction negative (just water) and positive extraction controls were included; these controls consisted of DNA alicutas (10ng/ml) of the *M. bovis* CIP 105050 strain.

2.4 Primers, biotinylated capture oligonucleotides and probe design.

The objective was to develop three independent tests for a multiple and simultaneous detection of: 1st , the organisms which belong to the *Mycobacterium tuberculosis* (CMTB) Complex; 2nd , the organisms which belong to the *Mycobacterium avium* (CMA) Complex (including *M. avium subsp. paratuberculosis*) and 3rd, the most number of species belonging to the *Mycobacterium* genus, except those ones belonging to CMTB and CMA which will be named NTM (non tuberculosis Mycobacteria).

Primers and TaqMan probes were selected based on alignments of the ITS region (16S-23S rDNA internal transcribed spacer (Roth et al., 1998)) with sequences from the National Centre for Biotechnology Information (NCBI) database. DNA sequences alignments were performed using the ClustalX program (http://www.ebi.ac.uk/clustalw/, European Bioinformatic Institute) and were made to investigate interspecies and intraspecies variations. The alignments include multiple stains of all species of the *Mycobacterium tuberculosis* Complex, the *Mycobacterium avium* Complex and several species of atypical mycobacteria such as *Mycobacterium massiliense*, *Mycobacterium abscessus*, *Mycobacterium vaccae*, *Mycobacterium fortuitum*,

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Mycobacterium gordonae, *Mycobacterium smegmatis, Mycobacterium kansasii* and *Mycobacterium chelonae.* An NCBI BLAST search was performed to check the specificity of the DNA sequences of primers and probes. Primers and probes were designed with the help of the Primer Express software (Applied Biosystems, Foster City, USA) and were checked with Oligo-Analyzer 3.0 (http://biotools.idtdna.com/analyzer), an online service of IDT Biotools (Coralville, Iowa), to ensure minimal selfcomplementarity and to prevent the presence of secondary structures. The sequence and properties of the oligonucleotides used are shown in Table 2. The complex-specific identification and primer and probe sets (detectors) were devised to simplify the assay. Thus, the three detectors share the UNIMYC-319R reverse primer. The primers and probes were selected after alignment of the micobacterial ITS sequence encompassing the 3´ end of the 16s rDNA and the 5' end of the 23s rDNA. Specific amplification was obtained by using the primers and probe combinations showed in Table 2. Primers and probes were synthesized at Applied Biosystems (Cheshire, UK).

The biotinylated capture oligonucleotides Ec16S.1390 (3´Biotin-aaaaaTTGTACACACCGCCCGTCA 5') and Mb23S.44n (3´ Biotin-aaaaaTCTCGATGCCAAGGCATCCACC 5´) (Roth et al., 1998) located in positions 156.664 to 156.682 (5' end of the 16s gene) and 157.137 to 157.116 (3' start of the 23s gene) on the genome sequence of *M. bovis* AF2122/97, respectively, were used to capture de ITS region of the *Mycobacterium* genus and were synthesized at Eurogentec (Seraing, Belgium).

2.5 Real-time PCR analysis

DNA detection was performed by amplification using the real-time 7.500 Sequence Detection System (Applied Biosystems, Foster City, USA). Reactions were carried out in duplicate in 96-well optical plates. The 25 µl reaction mixtures contained 12.5 µl of TaqMan Master Mix (which includes the heat-activated AmpliTaq Gold enzyme, Appied Biosystems, Foster City, USA), 300 nM of each specific primer and 300 nM of each MGB probe, and 3 µl of DNA. All the reactions included an internal positive control (IPC) as the inhibition detection system commercialized by Applied Biosystems *(TaqMan® Exogenous Internal Positive Control*

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Reagent, PN 4304437). In the same way, 2 non-amplification negative controls (No amplification Control, NAC, with no DNA problems and with DNA exogenous of the cutoff IPC), 2 negative controls with no DNA problems and two positive controls (including 1ng of the *M. Bovis* CIP 105050 strain) were used.

The reaction conditions were as follows: 10 min at 95ºC for enzyme activation and 40 cycles of 15s at 95ºC and 1 min at 55ºC. Readings were automatically taken by software every cycle, and the logarithmic of the increment in fluorescence was plotted versus the cycle number. The threshold level was fixed at the same midexponential position for all runs.

2.6 Data analysis.

Data were analyzed using the Sequence Detection Software 2.0 (Applied Biosystems, Foster City, USA). The fluorescence emission baseline was calculated during the first 3-15 cycles and the default threshold (Ct) set at three times the standard deviation of the baseline measurement. The mean value and standard deviation from three independent experiments, each performed with duplicate reactions, were used to build the standard curves for serial dilution DNA samples. The calibration curves were automatically constructed by the SDS 7.500 software.

3. Results

3.1 Specificity

The specificity was assessed with DNA from 11 mycobacterial species (Table 1) and 7 other bovine pathogens. Several pathogens that may cause lymphadenitis, such as *Streptococcus bovis*, *Arcanobacterium haemolyticum* and *Actinomyces bovis*, were also included in this specificity testing. No nonspecific results were obtained in the genus *Mycobacterium*s specific Q-PCR, but only *Mycobacterium gordonae, kansasii, szulgai, gastri, ulcerans,* could be detected. The *M. tuberculosis*-specific Q-PCR, detected only *M. bovis*. The *M. avium*-specific Q-PCR recognized six isolates belonging to all three subspecies of *M. avium* and was negative for *M. scrofulaceum* and other species tested (Table 1).

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3.2 Analytical sensitivity

In order to establish the minimum detection threshold (or assay experimental sensitivity), reference curves for each studied Complex (MTC, MAC and NTM) were carried out. For each Complex, 10 fold dilutions serial dilutions were created in triplicate; they contained from 1 µg to 1 femtograms. For the three assays, the SDS 7.500 system (Applied Biosystems) was able to detect and differentiate up to 8 logarithmic magnitude orders, with a minimum detection threshold of 10 femtograms (2-3 genomic equivalents) for the MTC and MAC detection assays and 100 femtograms for the Genus *Mycobacterium* detection assay. Regarding the MTC, a calibration straight line was obtained with a 2.77 slope, a Ct= 34,36 interception in the minimum threshold (10 fg/ml) and one R^2=0.957. For all systems generated, a strong linear relationship between the log of the starting amount of DNA and the Ct values obtained. Moreover, the sensitivity and dynamic range of the three assays were not affected by the presence of concentrations of up to 10 µg of bovine genomic DNA (data not shown).

3.3. Clinical sensitivity

A total amount of 125 bovine samples, obtained from intradermal skin test positive animals, were tested by macroscopic inspection, culture, PCR identification of culture isolates and multiplex real-time TaqMan-based assay. Of the 125 samples, 75 showed lesions consistent with TB, confirmed through bacterioscopy and 50 were considered to have no visible lesions (NVL) with negative bacterioscopy. The samples with lesions were later classified according to their pathological status in *incipient lesions* (n=44) confirmed by means of bacterioscopy (Ziehl-Nielsen), showing only unilateral damage of the retropharyngeal lymph node and *generalised lesions* (n=31) with different stages of evolution (from caseous lesions scarcely spread to granulomatous lesions which showed chronic calcification. Once the samples were cultured, 36 positive cultures were obtained from the samples classified as NVL (36/50), 33 in incipient lesions y 28 in widespread lesions. There was no positive culture in 14 samples, in spite of the fact that they showed compatible lesions and positive bacterioscopies. If we consider the combination of confirmed anatomic pathology with bacterioscopy and culture as

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the gold standard, we would have 97 animals positive to TB and 28 non-conclusive. This determines a culture sensibility of 77.6% (97/125). No mycobacteria other than *M. bovis* were detected by culture.

By applying the molecular detection system and using the conventional extraction system (Tri-Reagent), we detected 22 positive samples (22/36, 61.11%) to the MTC detector in the case of the NVL culture positive samples, 35 (31/44, 79.44%) in the samples with initial lesions and 25 (25/31, 80.64%) in the group of the widespread lesions, which determines a total sensitivity of 65.6% (82/125). Taking into account the field diagnosis as well as the bacterioscopy, we could consider 111 animals true positives (36 NVL positive culture +75 animals with compatible lesions and positive bacterioscopy), which determines a sensitivity of 73.87% (82/111). No Q-PCR positive samples were detected in the NVL group with no positive culture.

By applying the automated extraction system based on SC, we detected a decreasing sensitivity of up to 47.74% (1.47 times lower), with only 10 (10/36, 27.27%) positive samples in positive NVL/culture, 20 (20/44, 45.45%) in incipient lesions and 23 (23/31, 74.19%) in widespread lesions (Table 3). Organisms belonging to MAC or NTM were not detected, neither by means of a culture nor a Q-PCR process.

The sensitivity was positively correlated to the mean Ct for each kind of samples (Table 3 and Figure 1), with higher Ct in the samples with widespread lesions and lower in the NVL samples, as a clear indicator of the bacterial charge in each group of lesions. Along with this, the standard deviation in each group of lesions became higher, regardless of the kind of extraction system, indicating thus, great irregularity in the bacterial charges of each group.

4. Discussion

The purpose of this study was to develop, by using a multiplex real time PCR approach, a high-quality post-mortem diagnostic system applicable directly to bovine clinical specimens, in the context of an experimental abattoir epidemiological surveillance program, allowing a rapid and accurate identification of the infectious agent and/or confirmation of the field diagnosis. In this regard three independent quantitative PCR methods were designed and optimized.

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This objective was fulfilled in the case of the MTC and MAC organisms whereas the detection capacity of the genus was limited to 5 atypical mycobacteria species: *Mycobacterium gordonae, kansasii, szulgai, gastri, ulcerans*.

The magnitude of the detected detection thresholds (around 2-3 genomes) is similar to the ones detected in previous studies (Rodriguez et al., 1995; Taylor et al., 2001; Wards et al., 1995), while the detected experimental sensitivity (73.87%) is slightly higher than in previous studies, where it was around 70% (Liebana et al., 1995; Taylor et al., 2001; Vitale et al., 1998; Zanini et al., 2001); however, the detected sensitivity depending on the kind of lesion detected was not specified in any of the previous studies.

The sensitivity detected in relation to the culture can be considered similar to the one detected in previous studies carried out on the same population and kind of samples (Gonzalez Llamazares et al., 1999). The worst sensitivity detected using the robotic system (just the 48.18%) in comparison with previous studies (Roring et al., 2000; Taylor et al., 2001) is a disadvantage in relation to the general expected achievement and sensitivity of the SC manual process. This can indicate a lack of accuracy of the robotic system in comparison with the SC manual procedure. However, the Tri-Reagent extraction procedure, even though it is a manual method, it is a simple and rapid system that offers high sensitivity, completing an extraction of 24 samples in less than an hour. The different stages of the detected sensitivity (58.33% in NVL, 75% in the samples with initial lesions and 80.64% for the widespread lesions, by using the conventional extraction method), in relation to the pathological state of the animal, were previously described (Norby et al., 2004) for other diagnostic techniques and this fact could be useful when applying the molecular technique, being, in this particular case, useful in animals with lesions compatible with TB. The correlation of the different detected sensitivities with the Ct, is a clear reflection of the amount of bacteria which are present in each kind of samples and they also indicate the possibilities of the system for each kind of sample.

So, this approach offers three advantages: (i) the assay is fast (within two days for a minimum of 48 samples), (ii) the assay has a moderate sensibility and is extremely specific, as shown in

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samples NVL and AFB negative and (iii) it allows the detection of several mycobacterium species of veterinary interest (such as *M. bovis* and *M. avium subs. paratuberculosis*).

The addition of the IPC measured by a parallel real-time PCR assay completed the quality assurance of the diagnostic assay, as it allows the monitoring of booth the sample manipulation procedures and the presence of PCR inhibitors. Indeed, the DNA extraction procedure significantly affected the recovery rate of the nucleic acids, introducing a statistically significant bias of underestimation. Moreover, whereas the detection and amplification profile of the IPC reveals the presence of PCR inhibitors, the measurement of the sample recovery rate establishes the precise cuttoff of sensitivity for each negative sample.

In conclusion, our multiplex real time PCR system represents a fully controlled, fast, high-throughput diagnostic tool for the rapid detection of *Myobacterium* presence directly in animal clinical specimens, which could be an useful tool in the context of bovine tuberculosis abattoir surveillance programs and granuloma submission programs.

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Figure legends

Figure 1. Ct value versus DNA extraction system/pathological status groups plot. Extraction system: Sol= Guanidinium thiocyanate DNA purification, SC= DNA selective capture systems; Pathological status: NVL= non-visible lesions, IN= Early infection, Gen= Generalized condition.

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Table 1. Reference strains used in this study and results of real-time PCR

Strain	Conventional PCR results [1]	Q-PCR results[2]
Mycobacterium. bovis CIP 105050	+/+	+/-/-
Mycobacterium avium subsp *avium* CIP 104244	+/-	-/+/-
Mycobacterium avium subsp *avium* CIP 105415	+/-	-/+/-
Mycobacterium avium subsp *silvaticum* CIP 103317	+/-	-/+/-
Mycobacterium avium subsp *paratuberculosis* CIP 103963	+/-	-/+/-
Mycobacterium avium subsp *paratuberculosis* CIP 103964	+/-	-/+/-
Mycobacterium avium subsp *paratuberculosis* CIP 103965	+/-	-/+/-
Mycobacterium gordonae CIP 104529	+/-	-/-/+
Mycobacterium gordonae CIP 64.27	+/-	-/-/+
Mycobacterium kansasii CIP 104589	+/-	-/-/+
Mycobacterium. fortuitum CIP104534	+/-	-/-/-
Mycobacterium fortuitum subsp *fortuitum* CIP 104595	+/-	-/-/-
Mycobacterium abscessus CIP 104536	+/-	-/-/-
Mycobacterium flavescens CIP 104533	+/-	-/-/-
Mycobacterium scrofulaceum CIP 105416	+/-	-/-/-
Actinobacillus pleuropneumoniae CIP 100916	-/-	-/-/-
Actinomyces bovis CIP 103258	-/-	-/-/-
Arcanobacterium haemolyticum CIP 103370	-/-	-/-/-
Bordetella bronchiseptica CIP 102314	-/-	-/-/-
Corynebacterium bovis CIP 54.80	-/-	-/-/-
Haemophilus influenzae CIP 100032	-/-	-/-/-
Streptococcus bovis CIP 102302	-/-	-/-/-

[1] Molecular test based in 16S rRNA genus specific and MPB70 MTC specific markers (Liebana et al., 1996).

[2] Multiplex Q-PCR for MTC, MAC and NTM.

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Table 2. Oligonucleotide primers and probes.

Oligonucleotide	Sequence (5´→3´)	Target/sequence
Forward primers		
CMTB-224F	ACAAAGTTGGCCACCAACACA	CMTB / ITS
CMA-228F	CAAATGATTGCCAGACACACTATTG	CMA / ITS
GENMYC-243F	ACTATTGGGTCCTGAGGCAACA	*Genus*[a] / ITS
Reverse primer		
UNIMYC-319R	AAACACCACACCCCACCACC	Universal R / ITS
MGB Probes		
MGB-CMTB-283	FAM-TGTTGTCCCACCGCCT	CMTB / ITS
MGB-CMA-258	TAMRA-TGAGACAACACTCGGTC	CMA / ITS
MGB-GENMYC-285	NED-TGTCCCACCATCTTG	*Genus*[a] / ITS
Capture probes		
Ec16S.1390	Biotin-aaaaaTTGTACACACCGCCCGTCA	*Genus* / 16s rDNA
Mb23S.44n	Biotin- aaaaaTCTCGATGCCAAGGCATCCACC	*Genus* / 23s rDNA

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Table 3. Clinical sensibility (%) and *Ct* value in by extraction system and pathological group. .

Pathological status	NVL		Early infection	Generalized condition	Overall sensibility
Nº of samples/status	50		44	31	
Culture sensibility	72% (36/50)		75% (33/44)	90% (28/31)	77.6% (97/125)
Q-PCR	NVL Culture negative	NVL culture positive			
Conventional extraction sensibility	0 (0/14)	61.11% (22/36) [1] *30.62 (±1.12)* [2]	79.74% (35/44) *27.62 (±4.17)*	80.64% (25/31) *22.59 (±5.7)*	65.6% (82/125)
SC extraction sensibility	0 (0/14)	27.77% (10/36) *31.48 (±3.38)*	45.45% (20/44) *30.03(±2.3)*	74.19% (23/31) *26.18 (±6.31)*	42.4% (53/125)

[1] Sensibility (number of positive samples/number of samples by lesion type).

[2] *Ct value ± Std. Dev.*

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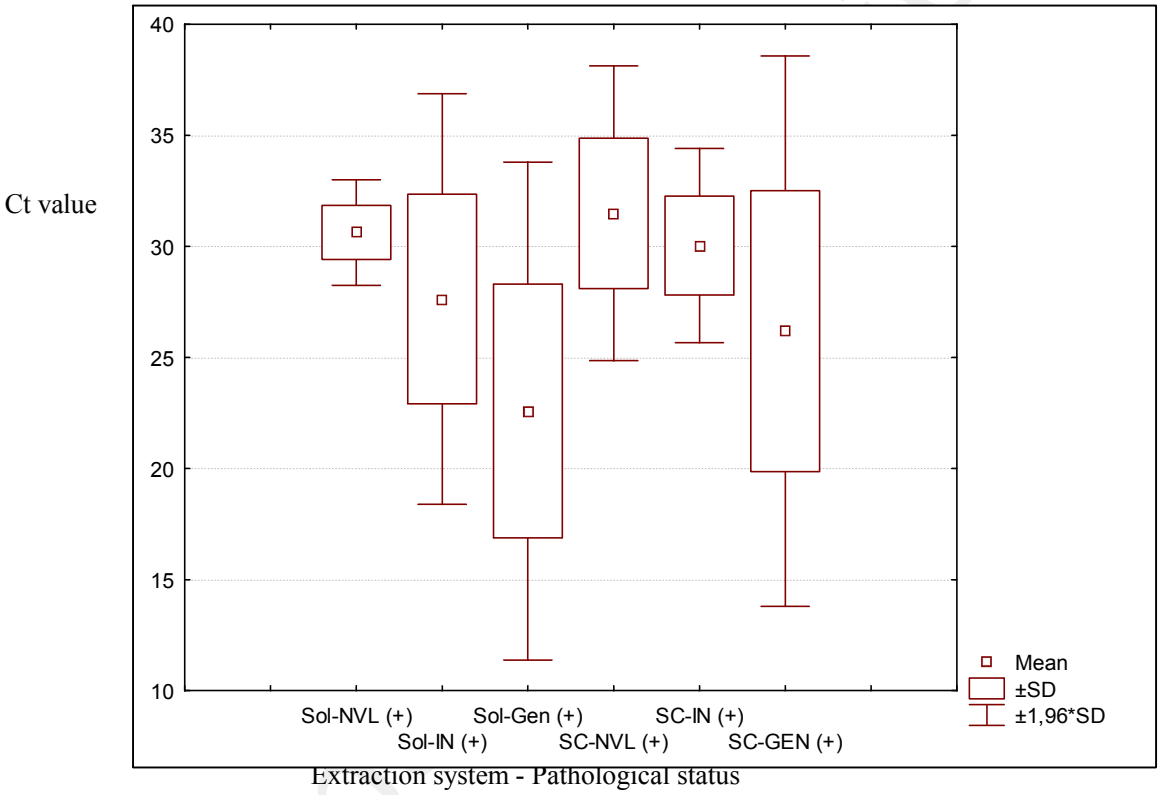
Figure 1. Ct value versus DNA extraction system/pathological status groups plot. Extraction system: Sol= Guanidinium thiocyanate DNA purification, SC= DNA selective capture systems; Pathological status: NVL= non-visible lesions, IN= Early infection, Gen= Generalized condition.