A Sensitive and Specific Duplex Real-Time PCR Assay Targeting Insertion Elements IS1081 and IS6110 for Detection of *Mycobacterium bovis* in Lymph Nodes of Cattle

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Abstract: The development of a reliable and rapid screening test for detection of *Mycobacterium bovis* (*M. bovis*) helps to control of bovine tuberculosis and preventing zoonotic infections. This study aimed to evaluate a sensitive and specific assay for detecting *M. bovis* DNA in lymph nodes with lesions suggestive to tuberculosis taken from slaughtered cattle. A duplex real-time PCR assay was developed for the identification of *M. bovis* targeting insertion elements (IS) IS1081 and IS 6110 in one internally controlled reaction. *M. bovis* DNA extraction protocols from tissue samples was evaluated. The specificity and sensitivity of the assay were evaluated for detecting serial dilutions of reference Mycobacteria strains as well as form spiked lymph node homogenate. Results revealed that microscopical examination of 600 lymph nodes with tuberculous-like lesion for detection of AFB showed a detection rate of 96.6%, compared to 98% *M. bovis* with duplex real-time PCR. The reproducible detection limit of the IS1081-PCR was 10 *M. bovis* cells/ml and the IS6110-PCR was 100 *M. bovis* cells/ml. Besides, both primer set of PCR protocol could detect 20 *M. bovis* cells/ml in spiked lymph node tissue. The assay was evaluated on 19 bacterial strains and was determined to be 100% specific for *M. bovis*. In conclusion, we suggest that the IS1081-PCR is a good candidate assay for routine screening of cattle lymph nodes and other tissue for *M. bovis* infection. Future validation of this assay for monitoring BTB in humans and animal species will be valuable for guiding public health policy.

Key words: *M. bovis*, IS1081, IS6110, Lymph nodes.

1. INTRODUCTION

Mycobacterium bovis (M. bovis) is the causative agent of bovine tuberculosis (BTB) which affects cattle and a wide range of other mammals, including humans. (Dye et al. 1999). BTB is considered one of the most important zoonotic diseases known to humans. WHO, in conjunction with FAO and OIE, classified BTB as a neglected zoonotic disease (Michel et al. 2009). Infection of human hosts with *M. bovis* can result from zoonotic exposure to TB infected animals and/or consumption of unpasteurized dairy products and meat products (Allix-Be`guec et al. 2010).

Tuberculosis in cattle and other domestic animals is above all caused by two members of *Mycobacterium tuberculosis* complex (*MTC*): *M. bovis* and *M. caprae* (Prodinger et al. 2005). However, occasional occurrence of tuberculosis due to *M. tuberculosis* species with concurrent tuberculous lesions has been reported in cattle (Popluhar et al. 1974; Pavlik et al. 2005), and other animals (Pavlik 2006; Popluhar et al. 1974). In addition, Mycobacteria other than *M. bovis* are routinely isolated from tissues submitted for diagnostic culture (OIE 2001). Aerosol exposure to *M. bovis* is considered to be the most frequent route of infection of cattle, but infection by ingestion of contaminated material also occurs. After infection, nonvascular nodular granulomas known as tubercles may develop (Cousin 2001). Characteristic tuberculous lesions occur most frequently in the lungs and the retropharyngeal, bronchial and mediastinal lymph nodes. Lesions can also be found in the mesenteric lymph nodes, liver, spleen, and in other organs. These lesions are visually detectable at slaughterhouses and should be followed by confirmation and identification of mycobacterium agent by other techniques (OIE 2009).

Detection of bovine tuberculosis in cattle and other susceptible animal species is often made on history, clinical and necropsy findings, tuberculin skin tests and abattoir meat inspections. Definitive diagnosis is made on culture with morphological appearance and biochemical tests for differential identification of members of genus Mycobacterium in most clinical laboratories (Kent & Kubica 1985). However, these conventional methods are time consuming and are difficult to assimilate into precise identification of closely related species and difficult to identify species. Genotypic assays molecular using biological techniques like PCR that use specific genetic elements have shown promise as alternative diagnostic tools (Rodriguez et al.1995, Kirschner et al. 1993; Cousin et al. 1991).

Genotyping of *M. bovis* probably lacks sufficiently informative methods. IS*6110* restriction fragment length polymorphism (RFLP) typing has been considered a gold standard method for

differentiation of *M. tuberculosis* strains for a long time; this has provided only limited discrimination among *M. bovis* populations where the majority of the isolates harbor only one or few IS copies (Haddad et al. 2004). PCR-based spoligotyping (Kamerbeek et al. 1997) has been widely used to genotype *M. bovis* isolates (Haddad et al. 2004); it is highly reproducible and rapid and represents the first universally recognized typing system for *M. bovis* populations. However, studies performed on *M. bovis* isolates showed a limited discrimination power of this method (Haddad et al. 2001). Rapid identification of isolates to the level of *M. tuberculosis* complex and specific identification of *M. bovis* can be made by Gen Probe TB complex DNA probe or polymerase chain reaction (PCR) targeting 16S–23S rRNA; the insertion sequences (IS) IS6110 and IS1081 and genes coding for *M. tuberculosis-complex*-specific proteins and targeting a mutation at specific nucleotides in oxyR gene, pncA gene, gyrB gene and presence/absence of Regions of Difference (RDs) (Niemann et al. 2000; Huard et al. 2006; Shitaye et al. 2006;Taylor et al. 2007; Reddington et al. 2011).

The development of a reliable and rapid screening test that would be of great help in the control of the disease and in specific situation such as faster confirmation of bovine TB infection in slaughterhouse cases. Therefore, this study aimed evaluated real-time PCR protocols based on two targets IS1081 and IS6110 as a sensitive screening method and specific confirmatory test for bovine TB.

2. MATERIAL AND METHODS

2.1 Animals and postmortem examination:

A total of 600 lymph nodes with visible suspected tuberculous lesions were collected from 300 carcasses of cattle slaughtered at Sachsen-anhalt Abattoir, Stendal City, Germany at the period from February 2008 to January 2010. The prescapular, axillary, supra mammary, prefemoral, suprarenal, mesenteric, ileocaecal, popliteal, retropharyngeal, hepatic and pulmonary lymph nodes were inspected and incised in situ. Caseated, suppurative and granulomatous lymph nodes were considered suggestive for tuberculosis. Samples were collected aseptically in polyethylene bags and quickly delivered to the laboratory in ice for further investigation.

2.2 Microscopic Detection of Acid Fast Bacilli by Zeihl-Neelsen stain.

Collected specimens from each macroscopic tuberculous-like lesion were examined for the presence of Acid-Fast Bacilli (AFB) in direct smear films was prepared from tissue exudate using Ziehl-Neelsen staining according to Wentworth (1987).

2.3 DNA Extraction from Lymph nodes:

a) Cetyl-trimethyl-ammonium-bromide (CTAB) Extraction:

Tissue specimens from suspected lymph nodes were homogenized in a tissue-grinding mortar with 10 ml of sterile saline for isolation of *M. bovis*. Genomic DNA of Mycobacteria was extracted by from tissue homogenate by acetyl-trimethyl-ammonium-bromide (CTAB) method described by Van Soolingen et al. (1991), which included a combination of chloroform/isoamylalcohol and isopropanol for extraction and precipitation of the DNA. After extraction, nucleic acid concentrations were measured by spectrophotometer (Du640 Photometer, Beckman Coulter GmbH, Krefeld, Germany) at 260 and 280 nm. According to calculation of Ravva and Stanker (2005), they calculate 5.1 fg of Mycobacterium DNA equal genome copy of one cell.

b) DNA extraction from lymph node samples using High Pure PCR Template Preparation kits (Roche) with modification:

400 µl of lysis buffer was added to 1gm lymph node sample in disruption tube containing beads, followed by adding 80 µl protinase k, followed by mechanical mixing in ribolyser at 6.5 ms^{-1} for 4x45 sec (Hybaid, Ashford, United Kingdom). The samples was incubated immediately at 56^{o}C overnight, incubated at 95 ^{o}C for 15 min to kill Mycobacterium and centrifuged at 5000 *xg* for 5 min. 200 µl of supernatant were mixed with 5 µl lysozme solution, and the sample was incubated in thermomixer (Eppendorf) at 37 ^{o}C and 550 rpm for 15 min. Then 300µl binding buffer was added. The samples incubate immediately at 70 $^{\circ}$C for 10 min, spined and the spined solution was add to the DNA binding columns provided by High Pure PCR Template Preparation kits (Roche) and processed as described by the kit manufacturer's procedure. Finally, the DNA template was eluted in 100 µl of the elution buffer and 5 µl aliquots were used as template in PCR-protocol.

2.4. Development of real-time PCR protocol to detect *M. bovis:*

The detection of Mycobacterium DNA by real-time PCR was performed based on two primer set. The first one based on IS1081 which present as multiple copies (6 copies) in Mycobacterium gene. The primer designed as MTC IS1081 F (FAM-5`-CTC TCG ACG

TTCA TCG CCG-3`) and MTC IS1081 R (5`- TGG CGG TAG CCG TTG CGC-3`) while the probe is FAM-ATT GGA CCG CTC ATC GCT GCG TTC-BHQ1 and another primer set designed according to (Cleary et al. 2003) based on IS6110 gene sequence as IS6 forward primer (5`-GGC TGT GGG TAG CAG ACC-3`), IS7 reverse primer (5`-CGG GTC CAG ATG GCT TGC-3`) and probe (FAM-5`-TGT CGA CCT GGG CAG GGT TCG-3`).

PCR reactions to detect mammalian β-actin were performed parallel with each sample. The primers ACT-F 5`-AGC GCA AGT ACT CCG TGT G-3`, ACT-R 5`- CGG ACT CAT CGT ACT CCT GCT T-3` and yakima yellow labeled probe ACT-5`-TCG CTG TCC ACC TTC CAG CAG ATG T-BHQ1 were designed according to Toussinat et al. (2007). β-actin was included as a positive control for the PCR reaction and to evaluate successful DNA extraction from tissue homogenate. Lack of amplification was assumed to indicate that the PCR reaction was inhibited. Primers were synthesized by BioTez-Berlin-Buch GmbH, Berlin, Germany. The probes were synthesized by Eurogentec S.A., Seraing, Belgium.

2.5Protocol of duplex real-time PCR:

Duplex real-time The PCR mixtures were prepared from QuantiTect Multiplex Norox MasterMix (Qiagen, Germany), forward and reverse primers (0.5 mM final conc.), (FAM, yakima yellow labelled probes) (0.2 mM final conc.), template DNA and adjusted to a final volume of 25ml with the addition of nuclease free dH2O.

The PCR reaction was performed in Stratagene thermocycler with the following program: Initial denaturation and activation of Taq-polymerase for 15 min at 95^{o}C followed by 45 cycles of 1 min at 94^{o}C, 1 min at 60^{o}C then cooling for 30 sec at 40^{o}C.

2.6 Analytical sensitivity of Duplex real-time PCR:

The analytical sensitivity of real-time PCR was determined of purified DNA from cultures of *M. bovis* BCG strain (bacillus Calmette-Gue´rin strain). The serial dilution of *M. bovis BCG* strain was prepared in sterile distilled water in broad range of DNA dilutions equivalent to 10^8 to 10^1 *M. bovis* cells/ml. These serial dilutions of DNA were 3-fold examined with IS1081 and IS6110 real-time PCR in internally controlled reaction with β-actin followed by calculation of mean Ct-values and standard deviations (SD).

2.7 Analytical specificity of real-time PCR protocol:

The real-time PCR protocols targeting IS1081 and IS6110 were evaluated for specificity to 19 Mycobacterium species reference isolates. DNA extracted from each isolate was diluted to two

dilutions, 5 ng/µl and 5 pg/µl as listed in table 1. All mycobacterium reference strains were isolated and identified by Friedrich-Loffler Institute (FLI), Jena, Germany.

2.8Preparation of spiked lymph node tissue homogenate samples:

Ten-fold serial dilutions of *M. bovis* BCG ranged from $2x10^1$ to $2x10^7$ cells/ml were prepared. Afterward, 1ml from each dilution was added and mixed to 1gm of lymph node homogenate samples which previous confirmed negative to mycobacterium with cultural and molecular techniques. Control positive and negative were included

3 RESULT

3.1 Sensitivity of duplex Real-time-PCR compared microscopic detection of *M. bovis:*

Results revealed that out of 600 lymph node sample with lesions suggestive to tuberculosis 580 (96.6%) was positive for AFB detected by microscopic examination of ZN stained smears. However, by duplex real-time PCR 588 (98%) was confirmed to *M. bovis* infection.

3.2 Analytical specificity:

The specificity of real-time PCR targeting IS1081 and IS6110 was evaluated to 19 strains of different Mycobacterial species. The real-time PCR targeting both IS1081 and IS6110 sequences showed negative result with all Mycobacterial species in two concentrations of DNA from each strain, 5ng/µl and 5pg/µl; while strong positive with *M. bovis* BCG was detected. Furthermore, β-actin internal control showed positive Ct-values with all Mycobacterial species including *M. bovis* BCG (table 1).

Table 1: Mycobacteria and non-mycobacteria analyzed for the determination of the specificity of real-time MAP-PCR

Species, Sub-species and Designation	**Type**	**Host species / Source**	**Target sequence**			
			IS1801		***IS6110***	
			Template concentration			
			5ng/µl	5pg/µl	5ng/µl	5pg/µl
M. avium subspecies avium						
(M128/2)	TS	Cattle	–	–	–	–
(01A1077/2)	FI-J	Cattle	–	–	–	–
(00A0720/2)	FI-J	Pig	–	–	–	–
(03A0910/2)	FI-J	Poultry	–	–	–	–
(03A2530/1)	FI-J	Poultry	–	–	–	–
M. avium subspecies hominisuis						
(01A0554/1)	FI-J	Pig	–	–	–	–
(01A1054/1)	FI-J	Human	–	–	–	–

(01A0255/1)	FI-J	Dog	–	–	–	–
M. bovis BCG (99A1119/1)		*a*	17.03	26.69	18.66	28.33
M. dierhoferi (M132/1)	TS	Environment	–	–	–	–
M. fortuitum (M134/1)	TS	Human	–	–	–	–
M. intracellulare (M136/1)	TS	*a*	–	–	–	–
M. nonchromogenicum (M433/1)	FI-J	Environment	–	–	–	–
M. abuense (03A0262/3)	TS	Human	–	–	–	–
M. phlei (M139/1)	TS	Phage	–	–	–	–
M. scrofulaceum (M 140/3)	TS	Human	–	–	–	–
M. smegmatis (M141/1)	TS	*a*	–	–	–	–
M. terrae (M142/B)		Cattle	–	–	–	–
M. tuberculosis (05A3246)	FI-J	*a*	–	–	–	–

TS = reference strains of species or subspecies, FI-J = field isolates from Germany cultivated in FLI Jena,ATCC = designation of type strains by the American Type Culture Collection, Rockville, USA, DSM = designation of type and reference strains of the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.
[a)] host species unknown

3.3 Analytical sensitivity:

The analytical sensitivity of real-time PCR based on IS1081 and IS6110 gene sequences in combination with β-actin showed a nearly similar reproducible detection level that was 10 *M. bovis* cells/ml IS1081,While; the reproducible detection limit of SI6110-PCR was 100 *M. bovis* cells/ml (table 2).

Table 2: Ct-values and standard deviation of real-time PCR based on IS1081 and IS6110 gene sequences with serial dilution of *M. bovis* cells.

M.bovis cells/ml	IS1081		IS6110	
	Mean Ct	SD	Mean Ct	SD
1.00+08	17.4	0.2	17.4	0.8
1.00+07	20.8	0.2	21.5	0.8
1.00+06	24	0.1	24.6	0.6
1.00+05	27.2	0.4	27.9	0.8
1.00+04	30.2	0.3	30.9	0.8
1.00+03	33.5	0.1	34.1	0.6
1.00+02	37.1	0.5	37.4	0.8
1.00+01	39.2	0.3	-	-

The calculation of amplification efficiency of IS6110 and IS1081-PCR showed strong linear relationship between Ct-values and the corresponding concentration of *M. bovis* cells in PCR.

The regression coefficient was (R^2=0.9985) while the regression coefficient in case of IS6110-PCR was (R^2=0.9971), (figure 1).

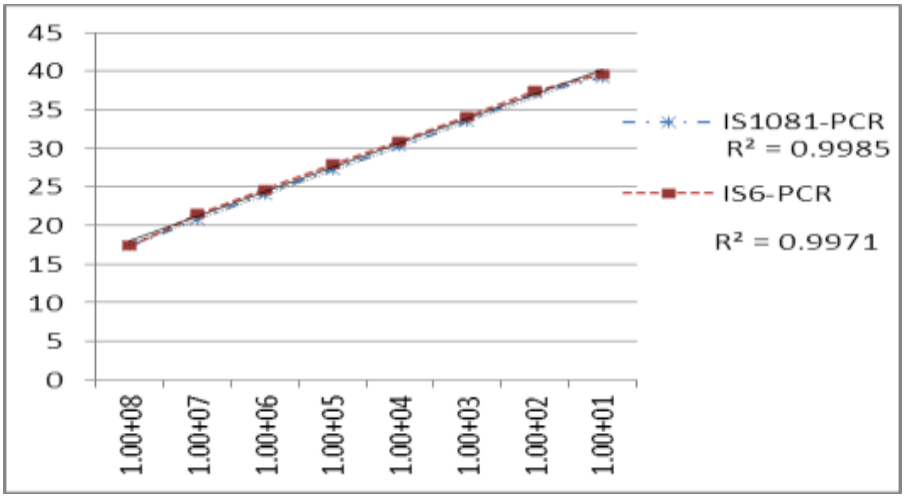

Figure 1: Semilogarthmic relationship between Ct-value and log concentration of *M. bovis* in spiked lymph node.

3.4 The accuracy of the assay for detection of *M. bovis* spiked LN tissue samples:

After DNA extraction from spiked lymph nodes samples using High Pure PCR Template Preparation Kit. By real-time PCR targeting IS1081 and IS6110 in combination β-actin internal. Up to 20 cells/gm Lnn could be detected (table 3).

Table 3: Results of direct detection of *M. bovis* in spiked lymph node using real time PCR based on IS1081 and IS6110.

Conc. Of M. bovis 1gm of spiked Lnn tissue	Ct-values	
	IS1081-PCR	IS6110-PCR
$2x10^7$	21.35	22.7
$2x10^6$	24.1	26.03
$2x10^5$	27.9	28.78
$2x10^4$	31.21	31.65
$2x10^3$	33.9	34.1
$2x10^2$	36.8	37.2
$2x10^1$	39.4	39.7

4 DISCUSSION

This study was included detection of *M. bovis* lymph nodes with visible lesions suggestive to tuberculosis in cattle slaughtered in a slaughterhouse. This constitutes a tentative diagnosis

for bovine tuberculosis that routinely performed during the meat inspection in slaughterhouses. However, the presence of caseous and/or calcified lesions and even lesions resembling tuberculous lesions may not always found to be of mycobacterial origin. Lesions can be caused by any other intracellular organisms or parasites that could mislead a veterinarian to consider the non tuberculous cattle as being tuberculous. Therefore, postmortem examination followed by bacteriological examination of suspected lesions in cattle is important tools to confirm BTB infection (Corner 1994). Accurate molecular detection enables to make definitive diagnosis of *M. bovis* (Haddad et al. 2004). It has been reported that PCR is 28 times more sensitive in the diagnosis of *M. tuberculosis complex* than traditional culture and direct microscopy (Romero et al. 1999). Molecular technique has the advantage of being fast. In addition, it is simple to implement and easy to adapt to any laboratory since it does not require sophisticated equipment or expensive reagents.

In this study, a high detection rate of AFB by microscopical examination of lymph nodes with tuberculous- like lesions was recorded. Although this assay is considered a tentative diagnosis and lacking of confirmation of BTB and its differentiation from other related mycobacteriosis, it is the most rapid and cost-effective screening method. This study results was in consistent with Asil et al. (2012) who sustains microscopy as a useful and accessible technique for detecting AFB. In contrary, *M. bovis* are often low in bovine specimens and they can be visualized by ZN only if a limited quantity (at least 5 × 104 mycobacteria/ml) of materials is present (Quinn et al. 1994). Result of ZN staining may also be affected by the sample taking technique during smear preparation as mycobacteria are not be evenly distributed in the tissue sample (Shitaye et al. 2006).

Tuberculosis in cattle and other domestic animals is above all caused by two members of *Mycobacterium tuberculosis* complex (*MTC*): *M. bovis* and *M. caprae* (Prodinger et al. 2005). However, *M. bovis* is the most prominent member of MTC associated with BTB infections. Results of this study revealed that 98% were confirmed to *M. bovis* by real-time PCR, indicating that *M. bovis* was the most predominated cause of BTB with high zoonotic potential. The negative results of 2% could be related to other type of mycobacteria or non-tuberculosis causes of detectable tuberculosis-like lesions.

In this study, results revealed that IS1081-PCR showed higher sensitivity than IS6110-PCR. In similar scale, our result showed high sensitivity than that reported by Thacker et al. (2011), the limit of detection of IS6110-PCR was 100 fg of *M. bovis* DNA (equal 20 *M. bovis*

cells). The IS110 PCR has been reported to detect M. bovis in PCR reaction (Shitaye et al. 2006; Ward et al. 1995). Reddington et al. (2011) developed a multiplex realtime PCR assay using a novel molecular targets to identify and differentiate between the phylogenetically closely related M. bovis M. bovis BCG and M. caprae.

However, the result of IS1081-PCR showed bit lower sensitivity than other detection limit one copy *M. bovis* reported by Taylor et al. (2007). The IS*1081* PCR is a realistic screening method for rapid identification of positive cases but the sensitivity of single copy methods; this is almost certainly due to the multi-copy nature of the target (Taylor et al. 2007).

Assay developed in this study could detect up to 20 *M. bovis* cells/ml in spiked lymph nodes. Numerous works have evaluated the use of PCR as a tool for diagnosing mycobacterial infection in various clinical samples. Some have detected *M. bovis* in milk samples (Antognoli et al. 2001), whereas others have detected *M. bovis* directly in bovine tissue (Roring et al. 2000; Taylor et al. 2007). Furthermore, Zanini et al. (2001) described a more effective use of this diagnostic tool applying the system in tissue samples with presence of gross lesions compatible with tuberculosis. Miller et al. (1997) demonstrated that PCR is a reliable technique for the identification of *M. bovis* in tissues embedded in paraffin in which *M. bovis* could not be cultured.

Efficient DNA extraction is crucial to the success rate of PCRs applied to such tissues. The extraction procedure should deliver effective lysis of Mycobacteria, good recovery of the DNA from a complex mixture of tissue debris and lastly, removal of PCR inhibitors. It was reported that the discrepancy between sensitivity of detection found with purified mycobacterial DNA and direct testing of field samples was due to limited mycobacterial DNA recovery from tissue homogenates rather than PCR inhibition (Taylor et al. 2007). This may be a cause of concern; they are irrelevant if primers and probes with sufficient specificity are not available. In the present study, efficient DNA extraction from lymph nodes was applied to overcome the PCR inhibitors by combination of commercial DNA extraction kits with bead beating technique and optimal lysis buffer. The use of commercial kits with lysis reagents was reported (Aldous et al. 2005). Silica based methods of DNA extraction have been widely evaluated and found to be one of the most efficient with columns generally more efficient than slurries ((Bouwman & Brown 2002). Combination of these various DNA extraction approaches also evaluated (Heginbotham et al., 2003). A number of studies have addressed the problem of initial processing of mycobacterial samples and a number of procedures as described (Afghani &

Stutman, 1996; Heginbotham et al. 2003; Tell et al. 2003; Mangiapan et al. 1996; Roring et al. 2000; Boom et al. 1990). Several of these studies have compared procedures, often with differing conclusions. The use of internal control in a real-time PCR reaction enables evaluation of DNA extraction and purification procedures and prevents misdiagnosis of *M. bovis* in clinical specimens.

CONCLUSION

The duplex real-time PCR assay described in this study is a diagnostic assay for the identification of *M. bovis* two diagnostic targets in one internally controlled reaction. The assay was evaluated on 19 bacterial strains and was determined to be 100% specific for the members of the *M. bovis* targeted. We suggest that the IS1081-PCR is a good candidate assay for routine screening of cattle lymph nodes and other tissue for *M. bovis* infection. Efficient DNA extraction is crucial to the success rate of PCRs applied to such tissues. Future validation of this assay for monitoring BTB in humans and animal species will be valuable for guiding public health policy and is important for the study of zoonotic BTB epidemiology.

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