Title

Evidence of *Mycobacterium tuberculosis* Complex bacteraemia in intradermal skin test positive cattle detected using phage-RPA

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Running Title

Detection of bacteraemia in TB reactor animals

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Abstract

Bovine tuberculosis is a zoonotic infectious disease caused by *Mycobacterium bovis* that affects cattle and can cause tuberculosis in a range of wildlife animals. A bacteriophage-based method combined with PCR (phage-PCR) has been recently used to detect and identify viable pathogenic mycobacteria in the peripheral blood mononuclear cells (PBMCs) of animals suffering from paratuberculosis. To adapt this method for the detection of *M. bovis* in blood, a new isothermal DNA amplification protocol using Recombinase Polymerase Amplification (RPA) was developed and was found to be able to detect *M. bovis* BCG within 48 h, with a limit of detection of approximately 10 cells per ml of blood for artificially inoculated blood samples. When blood samples (2 ml) from a Single Comparative Cervical Intradermal Tuberculin (SCCIT)- negative beef herd were tested, *Mycobacterium tuberculosis* complex (MTC) cells were not detected from any (45) of the blood samples. However when blood samples from SCCIT-positive animals were tested, viable MTC bacteria were detected in 66 % (27/41) of samples. Of these 41 animals sampled, 32 % (13) had visible lesions. In the visible lesion (VL) group, 85 % (11/13) had detectable levels of MTC whereas only 57 % (16/28) of animals which had no visible lesions (NVL) were found to have detectable mycobacteraemia. These results indicated that this simple, rapid method can be applied for the study of *M. bovis* infections. The frequency with which viable mycobacteria were detected in the peripheral blood of SCCIT-positive animals changes the paradigm of this disease.

Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis and forms part of *Mycobacterium tuberculosis* Complex group of pathogens. Bovine tuberculosis is a disease that affects primarily cattle, but can infect humans as well as a variety of other domestic and wild mammals.[1] Despite eradication schemes being in place since the 1950's, the UK has struggled to eradicate the disease; in fact the incidence of BTB outbreaks has increased and control measures continue to be a significant economic burden for this UK agriculture sector.[2] A major barrier to understanding and diagnosing *M. bovis* infection is that culture of these slow growing organisms is difficult, time consuming and often impracticable. This is especially true when undertaking epidemiological, infection or immunological studies compared to bacterial load.[3] In addition to the long incubation times required, a specific limitation of culture is that chemical decontamination used to inhibit the growth of competing bacteria also reduces the viability of the mycobacterial cells present in samples, reducing the sensitivity and reliability of culture as a method to detect and understand the disease.[4]

Essentially due to these difficulties, an area of bovine tuberculosis pathophysiology that is underreported is the development of disseminated infection and bacteraemia. It is known that *M. bovis* infection in an animal can have a number of possible outcomes ranging from elimination, self-limiting infection, localized lesions or a life-threatening systemic disease. The immune response following challenge is known to be complex, with variable responses occurring during natural infections with low numbers of organisms.[5] Bacteraemia occurs during post-primary dissemination in humans and it has been reported, although rarely, in cattle.[6] Recently a study in India reported the culture of *M. bovis* in the blood of apparently healthy cattle, which suggested *M. bovis* may be circulating in the blood of sub-clinically infected animals at higher levels than expected.[7] The paucity of information concerning this aspect of the disease means that it is not known whether bacteraemia will ultimately lead to active infection or clearance, nor how a positive SCCIT test result relates to the potential for bacteraemia.

Bacteriophage amplification technology was developed 20 years ago as a method to rapidly detect and enumerate slow growing pathogenic mycobacteria.[8] In addition it can be used as a tool to rapidly detect antibiotic resistance[9] and to investigate mycobacterial dormancy.[10] The assay detects the growth of broad host range mycobacteriophage, capable of infecting a wide range of both pathogenic and non-pathogenic mycobacteria. As the phage is specific for members of the *Mycobacterium* genus, and can only successfully replicate within a viable cell, a positive test result (a plaque) indicates the presence of viable mycobacterial cells in the original sample. The specificity of the detection event is then achieved by amplification of signature sequences from the plaque following the phage assay (see Fig 1;[11]). In contrast, direct PCR will detect DNA from both viable and non-viable cells and can be inhibited by components in the blood.[12] The combined phage-PCR method has been shown to be able to detect and enumerate *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in a range of matrices such as milk, cheese and blood,[11, 13, 14] but detection of *M. bovis* in clinical samples using this approach has not been described before.

It was already known that the phage-PCR assay could be used to sensitively detect *M. bovis* cells from liquid cultures[15] and that this method can be used to detect MAP cells in clinical blood samples.[16] The aim of these experiments was to design and optimise a phage-PCR method to allow detection of *M. bovis* cells in blood and to determine whether it could detect mycobacteria in SCCIT-positive animals.

Materials and Methods

Bacterial strains, bacteriophage and growth media

M. bovis BCG (Glaxo) was used to optimise the phage-PCR assay. Cells were cultured and maintained on Middlebrook 7H10 agar supplemented with OADC (Becton Dickenson, UK) without glycerol and liquid cultures were prepared in Middlebrook 7H9 media (Becton Dickenson, UK) containing OADC. When performing the phage assay the media was supplemented with $CaCl_2$ (2 mM final concentration; Sigma, UK). All cultures were grown at 37 ^{o}C without shaking to prevent clumping. For the phage assays the

Mycobacterium smegmatis strain used was mc^{2}155 (Lab21 Ltd, UK) and the bacteriophage used was D29 (Lab21 Ltd, UK).

Bacteriophage amplification assay

The phage assay (Fig. 1) and experimental controls were carried out as previously described.[15,16] Briefly, samples (1 ml) were mixed with bacteriophage D29 (100 µl; 1 x 10^8 pfu) in supplemented 7H9 Media and incubated for 1 h to allow the phage to infect any mycobacteria present in the sample. Phage that had not infected a host cell were then inactivated using a virucide (100 µl ferrous ammonium sulphate; final concentration 10 mM; Lab21 Ltd, UK). Samples were mixed thoroughly ensuring all sides of the container are covered and incubated for 5 min at room temperature. 1 ml of *M. smegmatis* cells (to form the bacterial lawn) was added to the sample which was plated in 7H10 agar (0.75 % final agar concentration). As experimental controls, a 1 ml sample containing approx. 1 x 10^3 cfu ml^{-1} *M. smegmatis* cells or 1 ml of medium alone were used as positive and negative controls, respectively for the phage assays. Plaques formed at the end of the assay were counted and the number taken to represent the number of cells that could be detected by the phage in the sample.

Molecular Identification of M. tuberculosis Complex cells

The identity of the mycobacterial cell detected was determined by extracting DNA from the centre of plaques (a maximum of 5) using agarose gel-DNA extraction columns (ZymoResearch, UK).[16] For PCR detection of MTC DNA, the IS*6110* PCR assay described by Eisenach[17] was used. In a 25 µl reaction volume, 1 µM of each primer was used with HotStarTaq Plus Master Mix Kit (Qiagen, UK). The PCR cycle conditions were 95 ^{o}C for 5 min followed by 30 cycles of 95 ^{o}C for 30s, 68 ^{o}C for 30 s and 72 ^{o}C for 1 min and a final extension at 72 ^{o}C for 7 min. Either IS*6110* or IS*1081*[18] was targeted for RPA detection using lyophilised reagents (TwistDx Ltd, UK). These were rehydrated using 37.5 µL TwistAmp Resuspension Buffer and 4 µL of 280 mM MgOAc. Template DNA (8.5 µL) was added to this and samples were incubated for 30 min at 39 ^{o}C. DNA extracted from *M.*

bovis BCG and sterile water were used as positive and negative controls, respectively. Amplified RPA products of approximately 220 bp were visualised using agarose gel DNA electrophoresis.

To determine the sensitivity of the RPA assay, agar extracted from *M. bovis* BCG plaques was mixed with agar extracted from plaques formed using the non-pathogen *M. smegmatis*. The number of *M. bovis* plaques added varied between 1 and 5, with the total number of plaques per sample being made up to 5 in each case using *M. smegmatis* plaques. DNA samples extracted from these mixtures were then subsequently tested using either the PCR or RPA assays described above.

Blood samples, preparation and isolation of Peripheral Blood Mononuclear Cells

For method optimisation experiments commercial heparinised sheep blood (Oxoid, UK) was used. The number of *M. bovis* BCG cells in laboratory cultures was enumerated using the bacteriophage assay as described above (data reported as pfu.ml^{-1})[8] and then cells diluted in PBS (10^5 to 10 pfu ml^{-1}). To allow uptake of bacterial cells by leukocytes the method of [19] was used. Briefly 1 ml of cell suspension was added to 9 ml fresh (< 1 d old) heparinised sheep blood and samples incubated at 37 °C on a rotating mixer for 4 h. After the uptake period, PBMCs were isolated from 2 ml blood using Ficoll-Paque Plus in Leucosep tubes (GE Healthcare Life Sciences, UK). The purified PBMC's were then lysed by osmotic shock by addition of supplemented 7H9 media to release any internalised bacteria. The phage assay was then performed to detect mycobacteria released from the PBMCs as described above.[11]

Clinical blood samples from the SCCIT-positive cattle were obtained directly after slaughter by a veterinary surgeon in the abattoir. These were Holstein cows from a farm in the South West of the UK within the High Risk zone. Blood samples from the control animals were obtained from a closed Holstein beef herd in a non-TB endemic area of the UK provided as superfluous material as part of an on-going herd health screening program under the Veterinary Surgeons Act. The study protocol was approved by the University of Nottingham, School of Veterinary Medicine and Science ethical review panel

prior to sample usage. Blood was collected in Vacutainer heparin tubes (Becton Dickenson) and 2 ml samples used for the isolation of the PBMCs which were tested using the phage assay using the same method described above.

Statistical analysis

Excel 2010 statistical add on package was used to initially determine whether the data were distributed normally or not, then GraphPad Prism was used for performing ANOVAs and post-hoc tests to determine significant differences in the data. Significance was determined at $p < 0.05$.

Results

Development of phage-PCR method for detection of M. bovis BCG in blood

An experiment was performed to determine whether the phage assay could also be used to detect *M. bovis* BCG cells present inside PBMCs but using a published method that allows mycobacteria to be taken up by leukocytes.[18,19] When using this method, if no incubation time is allowed for uptake, no mycobacteria are detected in the buffy coat layer demonstrating that the bacteria do not co-purify with the PBMCs (Table 1). When 10^5 *M. bovis* BCG cells were added to the blood, the number of plaques detected in the PBMC fraction was uncountable, indicating that the purified PBMCs contained more than 10^3 cells (Table 1). As the number of cells added to the blood was decreased, the number of plaques recorded in each sample also decreased until the number of cells detected in the PBMC fraction reached countable levels. This occurred when approximately 10^2 *M. bovis* BCG cells were added to the blood, at which point 65 (±13) plaques were recorded (Table 1) indicating an efficiency of uptake of the *M. bovis* BCG cells of at least 50%. If less than 10 *M. bovis* BCG cells were added to the sample, no cells were detected using the phage assay and this is consistent with the results gained when this method was used for the internalisation of MAP.[19] Given the efficiency of uptake, it was assumed that the limit of detection of this method was approximately 10

cells per sample. However it could be lower than this when naturally infected samples are tested since the cells are already internalised.

Molecular identification of M. tuberculosis Complex DNA

Previously we have used PCR to detect the MAP signature genetic elements present in DNA extracted from individual plaques,[14, 15] hence this approach was also applied here and the IS*6110* MTC signature sequence was amplified by PCR using a published method.[17] When the sample was composed of DNA extracted from 5 plaques arising from the detection of *M. bovis* BCG cells, the PCR assay was routinely able to detect the IS*6110* element (data not shown). However when the number of *M. bovis* BCG plaques varying between 1 and 5, was tested (Table 2), it was found that the PCR assay could not consistently detect the IS*6110* genetic element when only one or two *M. bovis* BCG plaques were present in the sample, suggesting that the PCR assay was not sensitive enough for reliable detection of low amounts of DNA. This is consistent with our observations when developing an assay for MAP, where it was found that a nested PCR more consistently detected low concentrations of IS*900* in samples that only contained one MAP-positive plaque.[16]

To improve the sensitivity of the molecular detection event, PCR amplification was replaced with DNA amplification using a published isothermal RPA method.[18] Using this method, both the IS*6110* and IS*1081* MTC signature sequences were always detected from DNA samples that contained the DNA extracted from five *M. bovis* BCG plaques. However, only the RPA IS*6110* primers were able to consistently amplify MTC DNA when agar was extracted from one *M. bovis* BCG plaque mixed with 4 *M. smegmatis* plaques (Table 2). Thus the RPA method using the IS*6110* primers was chosen for use for further experiments.

Detection of viable M. tuberculosis Complex cells in clinical blood samples

Blood samples were obtained from 41 SCCIT-positive animals after slaughter and the optimised phage-RPA method was used to detect any viable MTC cells present in the

sample. As a control 45 blood samples from cattle in herds with no previous history of *M. bovis* infection were also tested. From the 45 SCCIT-negative samples, six produced plaques (range = 1-11 per 2 ml blood; mean = 3.1), however none of these gave a positive IS*6110* RPA result (Table 3) indicating that no MTC DNA was detected in these samples. We have previously reported that the phage assay will produce low numbers of plaques from samples that do not give a positive result when interrogated using PCR.[15] However the plaque number in these samples is always low as seen here.

Within the SCCIT-positive group, 31 produced plaques. After DNA extraction, 27 of these samples produced a positive IS*6110* RPA result (Table 4) indicating that DNA from MTC bacteria was present. At post mortem 32 % (13/41) of the SCCIT-positive animals had visible lesions (VL; 3 Multiple, 10 Diffuse). Of the animals in the VL group, 85 % (11/13) had detectable mycobacteraemia based on the phage-RPA results (Table 4; Fig. 2). In samples from NVL animals only 57% (16/28) had detectable levels of MTC mycobacteria in their blood (Fig. 2). No post mortem results were available for the negative control group, as blood samples were taken from healthy animals.

Since the phage assay detects individual mycobacterial cells, the number of plaques detected is indicative of the microbial load in the blood sample. In the SCCIT-positive VL group, the average number of cells detected in phage-RPA positive samples was 30.4 (range = 8-63 pfu per 2 ml blood; Fig. 3). For the SCCIT-positive NVL group, the average number of cells detected in RPA-positive samples was 16.3 (range = 2-43 pfu per 2 ml blood; Fig. 3) and this number is significantly lower than the number of mycobacterial cells detected in the VL samples ($p < 0.05$) suggesting that the VL animals had a higher microbial load in the blood. Within the NVL group, the average plaque number for MTC-negative samples (phage-negative or phage positive, RPA-negative) was 2.3 (range = 0-16; Fig. 3) and this number was also significantly lower ($p < 0.05$) than the plaque number for the NVL-MTC positive samples (Fig.3).

Discussion

The bacteriophage-based detection method used here was originally developed as a commercial assay for the detection of human *M. tuberculosis* infections,[20] however more recently it has been shown to be a useful tool for the investigation of animal mycobacterial infections, such as Johne's disease.[13, 16] Combining phage-based detection with PCR-based identification methods allows both live/dead differentiation (the phage can only replicate in a viable cell) and also increases the sensitivity of PCR-based detection methods because the plaques formed allow efficient targeting of the genomic DNA released from individual cells which is preserved in the agar. The IS*6110* genetic element, although not specific for *M. bovis,* is extremely useful as a target for any DNA-based detection method, as it is present in multiple copies in the genome, allowing more sensitive detection of single cells. Although components of the PBMC fraction does not affect the phage-based assay, RPA was chosen as an alternative DNA amplification method since it is reported to be less susceptible to inhibition by blood,[21] and in this study the RPA detection of low concentrations of IS*6110* was found to be more reproducible than PCR-based amplification of the same signature sequences.

As we found that MAP cells were primarily located in the PBMCs of MAP-infected cattle,[16] we hypothesised that this may also be true for cattle suffering from *M. bovis* infection. Hence for this study the SCCIT-positive cattle were chosen simply to determine whether mycobacteraemia could be detected in the blood of these animals using the phage-RPA method. The fact that we detected viable MTC cells in the blood of 26 of the 41 animals tested is extremely interesting and demonstrates that SCCIT reactor animals (i.e those identified as infected by *M. bovis*) commonly contain detectable levels of viable mycobacteria within PBMCs. Although there is a lack of other reports describing the detection of *M. bovis* in the blood of cattle (apart from one study in 1977[6]), there are many publications that have demonstrated the detection of mycobacteria in blood for humans suffering from human tuberculosis by both culture and PCR.[22, 23]

In this study we have not used a signature sequence that is specific for *M. bovis*, rather we targeted the IS*6110* element which is specific for the MTC group of which *M. bovis* is a member. Targeting this well-characterised genetic element allows rapid and

267 sensitive detection which can be routinely used for a variety of applications, making the
268 IS*6110* genetic element a useful target despite the lack of specificity. Nonetheless as all
269 the animals tested were SCCIT test positive, and *M. bovis* was cultured from some
270 samples from this outbreak sent to the UK veterinary diagnostic laboratories (D Brewer,
271 APHA pers. comm.), it is a reasonable interpretation of the results gained in this study
272 that the cells detected in these samples were *M. bovis*. While this is true in the UK, other
273 members of the MTC that may be detected using this method in countries where *M.*
274 *bovis* is not prevalent. Interestingly none of the samples from control animals gave a
275 positive result with the IS*6110* RPA assay, but it must also be noted these samples were
276 obtained from live animals rather than at slaughter which may have influenced our
277 results gained. Samples from six of these animals produce plaques and this may be due
278 to the presence of a different type of mycobacterial cell, such as MAP, which is also
279 widespread within UK cattle populations.[16] In this study we did not test the samples for
280 the presence of other mycobacterial signature sequences, but this result emphasises the
281 fact that when using the phage-based detection method amplification of signature
282 sequences to confirm the identity of the cell detected is essential for accurate
283 interpretation of plaque results. In future studies it will be interesting to include more
284 specific *M. bovis* signature sequences as well as developing multiplex DNA amplification
285 assays such as we have previously described[14] to allow simultaneous detection of
286 different mycobacterial species.

287 Of the 41 SCCIT-positive samples tested, only 13 had visible lesions, suggesting these
288 animals had an advanced stage of infection and MTC cells were not detected in blood
289 samples from only two of these. This may have been because there was low bacterial
290 load in these blood samples which was not detectable by the phage assay. However the
291 overall pattern of results is consistent with the idea that animals with visible lesions have
292 a more disseminated infection and therefore perhaps it is not surprising to find that *M.*
293 *bovis* cells are actively replicating inside the PBMCs (phage D29 can only productively
294 infect actively growing cells[10]). It is also known that the sensitivity of identifying lesions
295 at post-mortem is low,[24] which may explain why 57% of the NVLs also had detectable

296 levels of viable mycobacteria in their blood. Despite this, there was a significantly lower
297 number of plaques detected in the MTC-positive NVL samples than was detected in MTC-
298 positive VL animals indicating a lower microbial load in this group of animals. This may
299 reflect the ability of the phage assay to detect early stages of infection in some animals,
300 but further studies on animals in the early stages of infection are required to support this
301 conclusion.

302 The level of mycobacteraemia in animals suffering from *M. bovis* infection is
303 underreported due to the difficulties associated with culturing *M. bovis* from clinical
304 samples, and therefore this aspect of the disease is not well understood. In human
305 tuberculosis and in other mycobacterial diseases, mycobacteraemia is considered as one
306 of the potentially most useful approaches to definitively diagnose tuberculosis.[23]
307 However culturing these organisms is very slow, expensive and insensitive, which limits
308 the use of culture as a Gold Standard when investigating and detecting infectious
309 diseases.[25] Although, several research groups have detected mycobacteraemia using
310 extraction of DNA and direct PCR, it is not certain whether the DNA detected came from
311 a viable cell or whether PCR inhibitors in blood limit the detection event.[26] The fact that
312 the phage-RPA method could rapidly and sensitively detect and enumerate viable
313 mycobacterial cells in clinical blood samples, providing results within 48 h, means that it
314 could be used to increase our understanding of *M. bovis* infections. Although parallel
315 testing with culture is needed, the phage assay may also aid the confirmation of BTB
316 infection in NVL cattle without the need for extended culture of samples. The blood from
317 the SCCIT test positive reactor animals was obtained from animals directly after
318 slaughter which may have impacted our finding compared to obtained samples from live
319 animals. To this end it would be interesting to know whether intermediate or non-SCCIT
320 reactor animals from an infected herd harbour mycobacteria in their blood in live
321 animals, and whether this relates to the probability of their progression to SCCIT-
322 positive status. Hence the phage-RPA provides a useful tool for monitoring levels of
323 mycobacteraemia in relation to the animals' immune response to disease, especially

during vaccine trials and will enable researchers to ask clinically important questions to further the understanding of this extremely difficult to control disease.

Conclusion

Here we show that the phage-RPA method can be applied to provide rapid, sensitive and specific detection of MTC cells in clinical blood samples. The finding that a number of SCCIT-positive animals with detectable levels of viable MTC cells present in these initial trial results were both encouraging and surprising, in particular the relationship seen between mycobacterial load in the blood and the animals' lesion status. More work is now required to develop species-specific RPA assays and to establish what sample volume is required to ensure sensitive detection of low levels of bacteria. In addition, studies specifically designed to investigate exactly how bacteraemia relates to disseminated infection are required.

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425

426 **Table 1. Detection of *M. bovis* BCG in PBMC's using the phage assay**

Approx. No. of BCG cells used to inoculate sheep's blood[1]	Avg. Number of Plaques detected (±SD)
10^5	Complete lysis
10^4	Confluent lysis
10^3	TNTC[2]
10^2	65 (±13)
10^1	17 (±4.5)
1	0
0	0
10^5 – no incubation	0

427

428 1 ml of *M. bovis* BCG cells was added to 9 ml of commercial sheep's blood and incubated
429 for 4 h at 37 °C. PBMCs were then isolated and any internalised mycobacteria detected
430 using the phage assay. For the "10^5 - no incubation" sample cells were added to the
431 blood and then then the buffy coat fraction immediately isolated with no time allowed for
432 internalisation. Results are reported as the average ±SD of 3 independent samples.

433

434 [1]The number of cells in the inoculum was determined using the phage assay and
435 represents the number of detectable cells added to the sample

436 [2]TNTC – Too Numerous to Count

437 **Table 2. Performance of different amplification methods to Detect MTC**

438 **signature sequences**

	Detection of MTC Signature Sequences in DNA extracted from plaques[1]		
No. of *M. bovis* Plaques[2]	**IS*6110* (PCR)[3]**	**IS*6110* (RPA)[4]**	**IS*1081* (RPA)[4]**
5	+	+	+
4	+	+	+
3	+	+	+
2	+/-	+	+/-
1	+/-	+	-

439

440 *M. bovis* cells were mixed with purified sheep PMBCs and then the phage assay carried
441 out to generate *M. bovis* plaques. *M. smegmatis* plaques were recovered from plates of
442 the routine positive control samples.

443 [1] Result reported on the basis of the results of three independent tests

444 [2] DNA was extracted from 5 plaques for each test sample, but the number of *M. bovis*
445 plaques used varied between 1 and 5; plaques formed following the detection of *M.*
446 *smegmatis* cells were added to samples to keep the volume of agar being tested
447 consistent (approx. 50 µl).

448 + : Denotes a positive amplification of target sequence for all samples (n=3); - :
449 Denotes no amplification of the target sequence (n=3); +/- : Denotes both positive and
450 negative amplification of the target sequence when three replicates were performed.

451 **Table 3. Detection of viable MTC bacteria in PBMCs isolated from a SCCIT-**

452 **negative herd**

Assigned Number	No. of Plaques	IS*6110* RPA
1	0	NA
2	0	NA
3	0	NA
4	0	NA
5	0	NA
6	0	NA
7	0	NA
8	0	NA
9	1	-ve
10	0	NA
11	0	NA
12	0	NA
13	0	NA
14	0	NA
15	0	NA
16	0	NA
17	0	NA
18	0	NA
19	0	NA
20	0	NA
21	0	NA
22	0	NA
23	0	NA
24	0	NA
25	0	NA
26	0	NA
27	0	NA
28	0	NA
29	11	-ve
30	2	-ve
31	3	-ve
32	1	-ve
33	0	NA
34	0	NA
35	0	NA
36	0	NA
37	1	-ve
38	0	NA
39	0	NA
40	0	NA
41	0	NA
42	0	NA
43	0	NA
44	0	NA
45	0	NA

453

454 NA – Not Applicable as no plaques were formed.

455 **Table 4. Detection of viable MTC bacteria in PBMCs isolated from SCCIT-**

456 **positive cattle**

Assigned Number	No. of Plaques	IS6110 RPA	Post-Mortem Results[a]
1	63	+ve	Diffuse VL
2	27	+ve	NVL
3	43	+ve	Diffuse VL
4	22	+ve	Diffuse VL
5	60	+ve	Diffuse VL
6	36	+ve	Diffuse VL
7	11	+ve	Diffuse VL
8	14	+ve	Diffuse VL
9	35	+ve	Multiple VL
10	15	+ve	NVL
11	14	+ve	NVL
12	25	+ve	NVL
13	43	+ve	NVL
14	26	+ve	Diffuse VL
15	3	+ve	NVL
16	4	+ve	NVL
17	5	+ve	NVL
18	20	+ve	NVL
19	7	+ve	NVL
20	0	NA	NVL
21	2	+ve	NVL
22	0	NA	NVL
23	16	+ve	Multiple VL
24	0	NA	Diffuse VL
25	8	+ve	Diffuse VL
26	0	NA	NVL
27	8	+ve	NVL
28	7	-ve	NVL
29	0	NA	NVL
30	24	+ve	NVL
31	16	-ve	NVL
32	0	NA	NVL
33	0	NA	NVL
34	0	NA	Multiple VL
35	0	NA	NVL
36	8	+ve	NVL
37	32	+ve	NVL
38	23	+ve	NVL
39	3	-ve	NVL
40	1	-ve	NVL
41	0	NA	NVL

457 [a] VL, visible lesion; NVL, non-visible lesions.

458 NA – Not Applicable as no plaques were formed.

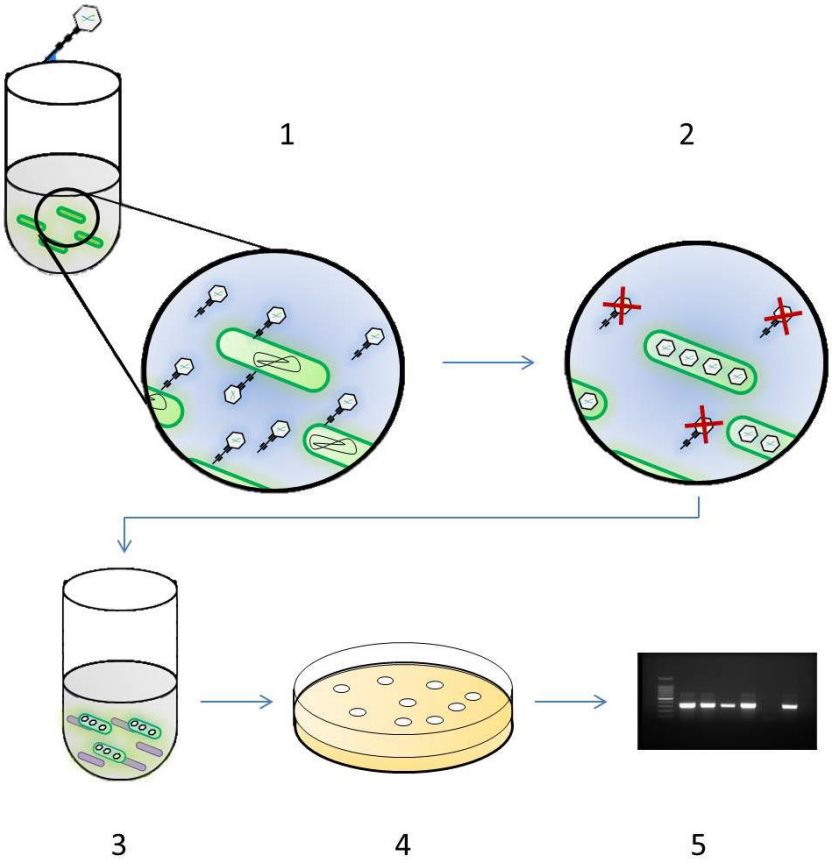

Fig. 1. Schematic of the bacteriophage amplification assay

To perform the phage assay, the mycobacteria first need to be isolated from the sample. In all these experiments PBMCs were purified and suspended in supplemented 7H9 medium that lyses the PBMCs and promotes efficient phage infection: Step 1: Isolated mycobacteria are incubated with phage D29 for 1 h to allow virus infection of mycobacterial cells present in the sample. Step 2: Extracellular phage that have not infected bacterial cells are inactivated by virucide. Step 3: Virucide is neutralized and fast-growing *M. smegmatis* are added to the sample which will form the bacterial lawn. Step 4: Samples are plated in soft agar and incubated overnight permitting lawn formation by *M. smegmatis.* Phage released from the infected mycobacterial cells present in the original sample are released and infect the *M. smegmatis* cells resulting in the formation of plaques. Step 5: Plates are inspected for plaques which indicate detection of a mycobacterial cell in the original sample. If plaques are present, DNA is extracted from the agar and presence of MTC bacteria is determined by RPA/PCR.

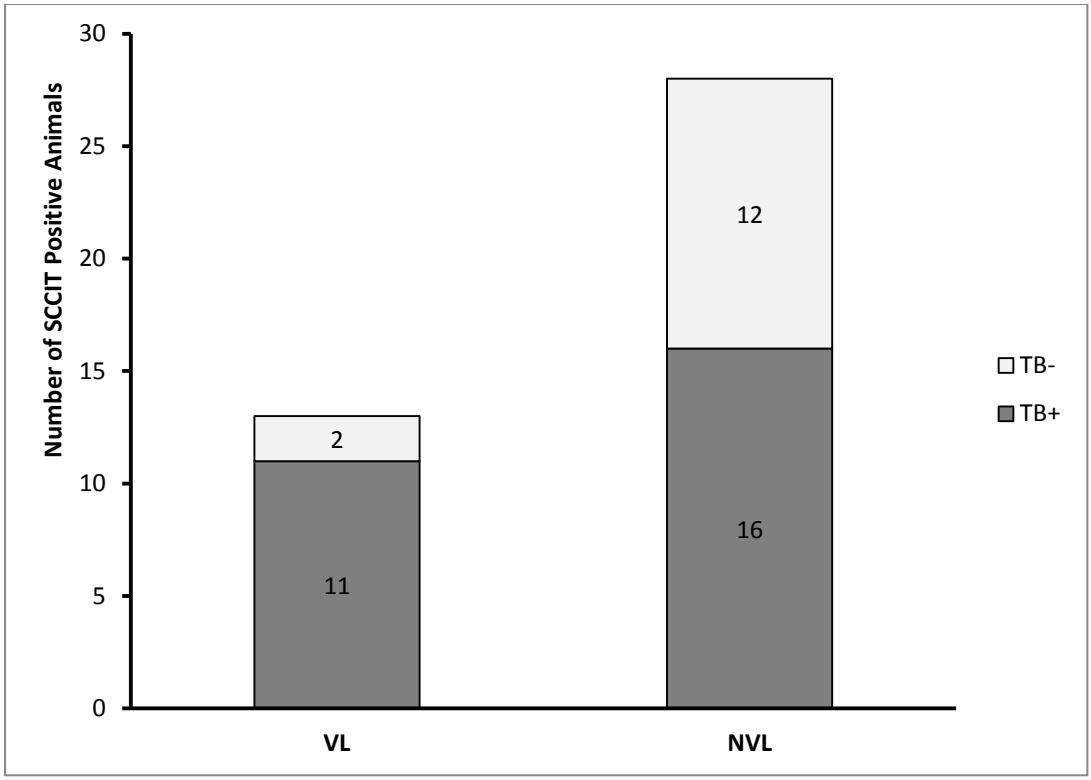

Fig. 2. Frequency of detection of MTC-bacteraemia in VL and NVL groups

Distribution of MTC bacteraemia-positive (Dark Grey) and MTC bacteraemia-negative (Light Grey) samples for animals classified as having visible lesions (VL) or non-visible lesions (NVL) at post mortem.

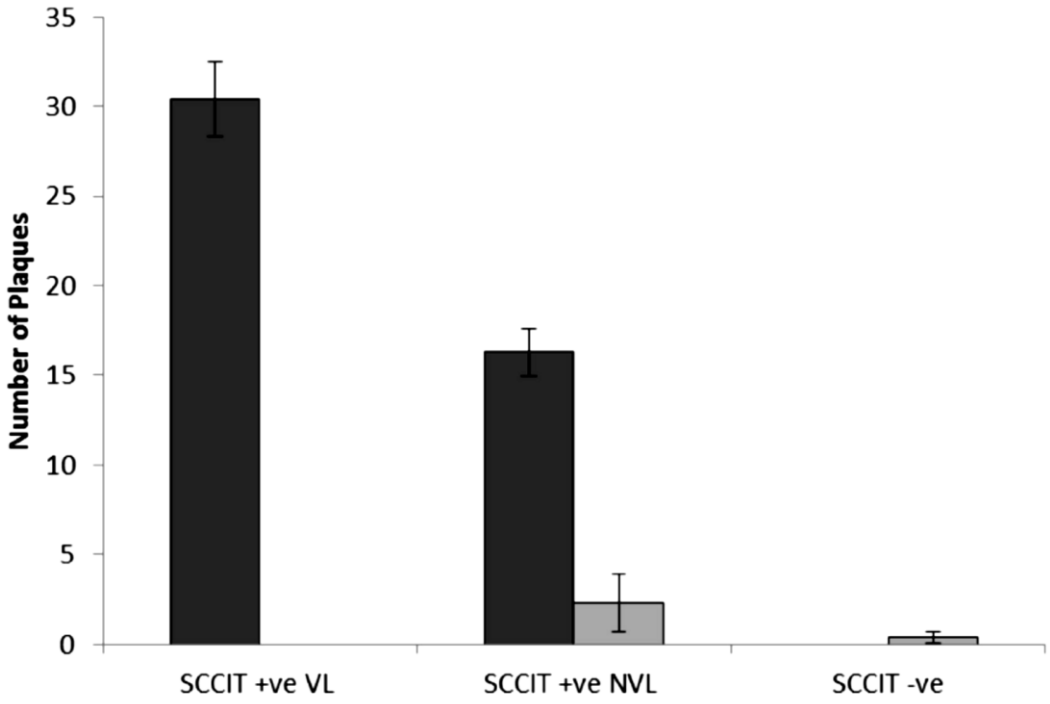

Fig. 3. Average plaque number detected for SCCIT positive and negative VL and NVL samples

Average number of plaques (±SD) formed per 2 ml blood sample for SCCIT positive animals that were classified as having visible lesions (VL; n=13) or non-visible lesions (NVL; n=28) at post mortem or SCCIT negative animals (n=45). Samples are further divided into those that gave a positive IS*6110*-RPA result (dark grey) and those that either formed no plaques or gave a negative IS*6110* RPA result (Light Grey). One-way ANOVA and Post-hoc Dunnetts-test was used to determine differences between these data sets.