frontiers

Improved culture medium (TiKa) for Mycobacterium avium subspecies paratuberculosis (MAP) matches qPCR sensitivity and reveals significant proportions of non-viable MAP in lymphoid tissue of vaccinated MAP challenged animals.

Tim J. Bull[1*], Tulika Munshi[1], Heidi Mikkelsen[2], Sofie Bruun Hartmann[2], Maria Rathmann Sørensen[2], Joana Sequeira Garcia[1], Paula M. Lopez-Perez[1], Sven Hofmann[1], Kai Hilpert[1], Gregers Jungersen[2]

[1]Institute of Infection and Immunity, St George's University of London, United Kingdom, [2]National Veterinary Institute, Technical University of Denmark, Denmark

In review

Submitted to Journal:
Frontiers in Microbiology

Specialty Section:
Food Microbiology

Article type:
Original Research Article

Manuscript ID:
234568

Received on:
02 Oct 2016

Revised on:
29 Nov 2016

Frontiers website link:
www.frontiersin.org

frontiers

Conflict of interest statement

The authors declare a potential conflict of interest and state it below

T. J. Bull and K. Hilpert declare their affiliation with TiKa Diagnostics Ltd. The company did not influence the design, conduction, interpretation or evaluation of this study.

Author contribution statement

GJ conceived and designed the experiments. TB co-ordinated culture experiments, analysed data and wrote the paper in collaboration with all authors. GJ, JSG, SBH, HM, MRS and TM performed experiments and analysed data. KH, PLP, SH contributed reagents and materials.

Keywords

Mycobacterium avium subspecies paratuberculosis, improved culture, quantification, qPCR, TiKa culture

Abstract

Word count: 262

The quantitative detection of viable pathogen load is an important tool in determining the degree of infection in animals and contamination of foodstuffs. Current conventional culture methods are limited in their ability to determine these levels in Mycobacterium avium subspecies paratuberculosis (MAP) due to slow growth, clumping and low recoverability issues. The principle goal of this study was to evaluate a novel culturing process (TiKa) with unique ability to stimulate MAP growth from low sample loads and dilutions. We demonstrate it was able to stimulate a mean 29 fold increase in recoverability and an improved sensitivity of up to 3 logs when compared with conventional culture. Using TiKa culture, MAP clumping was minimal and produced visible colonies in half the time required by standard culture methods. Parallel quantitative evaluation of the TiKa culture approach and qPCR on MAP loads in tissue and gut mucosal samples from a MAP vaccine-challenge study, showed good correlations between colony counts (cfu) and qPCR derived genome equivalents (Geq) over a large range of loads with a 30% greater sensitivity for TiKa culture approach at low loads (2 logs). Furthermore the relative fold changes in Geq and cfu from the TiKa culture approach suggests that non-mucosal tissue loads from MAP infected animals contained a reduced proportion of non-viable MAP (mean 19 fold) which was reduced significantly further (mean 190 fold) in vaccinated 'reactor' calves. This study shows TiKa culture equates well with qPCR and provides important evidence that accuracy in estimating viable MAP load using DNA tests alone may vary significantly between samples of mucosal and lymphatic origin.

Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

Does the study presented in the manuscript involve human or animal subjects: Yes

Please provide the complete ethics statement for your manuscript. Note that the statement will be directly added to the manuscript file for peer-review, and should include the following information:

- Full name of the ethics committee that approved the study
- Consent procedure used for human participants or for animal owners
- Any additional considerations of the study in cases where vulnerable populations were involved, for example minors, persons with disabilities or endangered animal species

As per the Frontiers authors guidelines, you are required to use the following format for statements involving human subjects: This study was carried out in accordance with the recommendations of 'name of guidelines, name of committee' with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the 'name of committee'.
For statements involving animal subjects, please use:
This study was carried out in accordance with the recommendations of 'name of guidelines, name of committee'. The protocol was approved by the 'name of committee'.

In review

If the study was exempt from one or more of the above requirements, please provide a statement with the reason for the exemption(s).
Ensure that your statement is phrased in a complete way, with clear and concise sentences.

All animal procedures were approved and controlled by the Danish National Experiments Inspectorate.

In review

1 *Original Research*

2 **Improved culture medium (TiKa) for *Mycobacterium avium* subspecies *paratuberculosis***
3 **(MAP) matches qPCR sensitivity and reveals significant proportions of non-viable MAP**
4 **in lymphoid tissue of vaccinated MAP challenged animals.**

5

6 Tim J Bull [1*], Tulika Munshi [1], Heidi Mikkelsen[2], Sofie Bruun Hartmann[2], Maria Rathmann
7 Sørensen[2], Joanna Sequeira Garcia[1], Paula M Lopez-Perez, Sven Hofmann[1], Kai Hilpert[1],
8 Gregers Jungersen[2].

9 **Affiliations**

10 [1] Institute of Infection and Immunity, St George's University, Cranmer Terrace, London SW17
11 0RE.

12 [2] National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870
13 Frederiksberg C, Denmark

14

15 **Running title**

16 Improved culture medium for MAP

17

18 **Keywords**

19 *Mycobacterium avium* subspecies *paratuberculosis*, improved culture, quantification, qPCR,
20 TiKa culture

In review

21

22 ***Corresponding Author: Tim J Bull**

23 Email: tim.bull@sgul.ac.uk; Tel: +44 (0)20 8725 0049

24

25

26

ABSTRACT

The quantitative detection of viable pathogen load is an important tool in determining the degree of infection in animals and contamination of foodstuffs. Current conventional culture methods are limited in their ability to determine these levels in *Mycobacterium avium* subspecies *paratuberculosis* (MAP) due to slow growth, clumping and low recoverability issues. The principle goal of this study was to evaluate a novel culturing process (TiKa) with unique ability to stimulate MAP growth from low sample loads and dilutions. We demonstrate it was able to stimulate a mean 29 fold increase in recoverability and an improved sensitivity of up to 3 logs when compared with conventional culture. Using TiKa culture, MAP clumping was minimal and produced visible colonies in half the time required by standard culture methods. Parallel quantitative evaluation of the TiKa culture approach and qPCR on MAP loads in tissue and gut mucosal samples from a MAP vaccine-challenge study, showed good correlations between colony counts (cfu) and qPCR derived genome equivalents (Geq) over a large range of loads with a 30% greater sensitivity for TiKa culture approach at low loads (2 logs). Furthermore the relative fold changes in Geq and cfu from the TiKa culture approach suggests that non-mucosal tissue loads from MAP infected animals contained a reduced proportion of non-viable MAP (mean 19 fold) which was reduced significantly further (mean 190 fold) in vaccinated 'reactor' calves. This study shows TiKa culture equates well with qPCR and provides important evidence that accuracy in estimating viable MAP load using DNA tests alone may vary significantly between samples of mucosal and lymphatic origin.

In review

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP) is an economically important pathogen (McAloon et al., 2016) causing Johne's disease in wide range of wild and domestic animals that has been linked as a zoonotic agent involved in the progression of Crohn's disease in humans (Gitlin et al., 2012). The ability of MAP to exist in a variety of phenotypes, some with a high resistance to killing (Grant and Rowe, 2004), has increased the importance of providing accurate quantitative estimates of viable counts when testing for the presence of this pathogen in food (Botsaris et al., 2016; Galiero et al., 2016; Ricchi et al., 2016), animal and human samples (Timms et al., 2016). MAP is widely accepted as a difficult organism to culture reproducibly and accurately, particularly at low loads (Hines et al., 2007). This is particularly relevant in early stages of MAP disease pathogenesis which are often interspersed with periods of low MAP shedding, presumably as a result of diminutive loads in tissues (Kalis et al., 2001). MAP culture requires specialist media supplements, grows only relatively slowly, aggregates during liquid phases of sample preparation forming various sized colonies and like other pathogenic mycobacteria is difficult to recover when plated on solid media in very low dilution (Harris and Barletta, 2001; Elguezabal et al., 2011). It is particularly adapted to intracellular persistence and is known to exhibit several phenotypes (Nazareth et al., 2015). Whilst detection is not necessarily indicative of clinical disease, identifying the presence and quantity of viable MAP provides an important marker of disease and infectious spreading potential, particularly relevant to optimising strategies of disease control at herd level and for individual assessments of treatment efficacies.

Quantitative detection of viable MAP by culture, particularly at low loads or from clinical tissue is thus considered challenging and as a consequence, molecular based detection systems

71 have been developed (Whittington, 2009). To validate molecular methods such as qPCR, 72 however, requires calibration that assumes accurate colony forming unit or viability count 73 estimations, efficient DNA extraction and DNA purification processing (Bull et al., 2003; 74 Elguezabal et al., 2011). Robust techniques for DNA extraction and purification have provided 75 means to specifically and reliably detect at least 100-500 genome equivalents which when 76 applied to adequate sample sizes can reduce the sensitivity to single log loads (Plain et al., 77 2014). The lack of culture correlation data however, has until now prevented accurate means 78 of estimating the true proportion of viable organisms in any sample tested.

79 In this study we have evaluated a new culture process that uses supplements able to stimulate 80 MAP growth. We show that for the first time, this generates sensitive, quantitative and reliable 81 MAP recovery and culture from infected animals. Using this as a comparator with molecular 82 methods we demonstrate that determining MAP presence by DNA based testing alone can 83 significantly overestimate viable MAP presence in immune-reactive lymphatic tissue.

84

85 MATERIALS AND METHODS

86 Media and antibiotics

87 Middlebrook 7H9 / 7H11 media and OADC supplement were obtained from Becton Dickinson, 88 UK, and Mueller Hinton broth from Merck, USA. All antibiotics were obtained from Apollo 89 Scientific, UK and chemicals from Sigma, UK unless otherwise stated. All mycobacterial 90 liquid cultures were set up in BACTEC MGIT 320 mycobacterial detection system which uses 91 barcoded tubes with 7 ml media and additional growth supplement (Becton Dickinson, UK).

In review

92 Animals, MAP challenge and vaccinations

93 49 Jersey calves (3 heifers, 46 bull) were enrolled in batches of 7 on a Danish Jersey dairy farm 94 with near zero prevalence of MAP infection as evidenced by several years of seroprevalence 95 monitoring through the Danish Paratuberculosis eradication program. Following inoculation, 96 all animals were housed in community pens with straw bedding in a secluded area of the farm. 97 As animals reached 6 weeks old, they were randomly assigned to a single Silirum® (CZ 98 Veterinaria) (N=1) immunization or two immunizations 4 weeks apart with one of two test 99 vaccines (N=2+2), or a saline sham-vaccination (N=2).

100 All calves were MAP challenged 3 weeks after the last vaccination which was administered as 101 three consecutive oral doses. The MAP challenge strain used was a clinical isolate Ejlskov2007 102 isolated from the faeces of a disease cow cultured in liquid Middlebrook 7H9 supplemented 103 with 10% OADC, 0.05% Tween 80 and 2 mg/ml Mycobactin J (MB7H9). A pool of Batch 104 190115 (7 g pelleted weight) and Batch 100315 (9.7 g pelleted weight) was thoroughly mixed 105 and re-suspended in 120 ml of MB7H9 media with 15% glycerol before aliquoting into 5 ml 106 vials (each containing 0.7 g pelleted MAP weight). To best estimate the number of MAP 107 genome equivalents per aliquot we used a qPCR directed against a single copy MAP gene 108 (FadE5) using primer pairs (5'-AAGTCGAACAGGAACTTGGG-3', 5'- 109 TCGAGAACATCTTCCACCTG-3') that had been previously shown to give accurate 110 evaluations at the concentrations expected. All samples were run in duplicate using 2.5 µl 111 DNA (5x pre-diluted) template, 12.5 µl QuantiTect SYBR green PCR kit (Qiagen, UK) and 112 0.125 µl of each primer (10 µM stock) in a total volume of 25 µl using a Rotor-Gene Q (Qiagen,

UK) PCR machine. PCR cycling included an initial denaturation at 95°C for 15 min, followed by 45 cycles of 95 °C : 30 sec, 62°C :60 sec. Data analysis was performed using the Rotor-Gene Q Series Software version 1.7. All aliquots were then frozen at -80 °C. One to three days prior to each block-inoculation, a vial was thawed, added to 15 ml sterile PBS, blended with single use sterile plastic homogenizer to resolve MAP clumps and refrigerated until use. On the morning of inoculation the material was re-suspended and aliquoted in 2 ml subsamples each containing 100 mg MAP. At the time of inoculation each tube was re-suspended in 800 ml warmed (37 °C) fresh milk and fed individually by calf drench. A total of three inoculations were given to each calf every second day. All calves were fed milk daily up to the time of inoculation to maintain the gastro-oesophageal reflex bypassing the rumen.

Sample testing

At 28 weeks post challenge, all animals were euthanized and necropsied. Approximately 8 cm intestinal tissue was collected from ileal and jejunal sites of each animal located at various distances 0, 25, 50, 150 and 250 cm from the ileocecal valve in the proximal direction. All samples included Peyer's patches were processed within two days of slaughter without prior freezing. DNA extraction method was as previous described (Park et al., 2014) with some modification. Samples of 100 mg tissue in 1.5 Milli-Q water were refrigerated 16-40 hours and centrifuged at 15,000 xg for 15 min at room temperature (RT) and the tissue pellet re-suspended in 360 µl Qiagen tissue lysis (ATL) buffer and 40 µl proteinase K (Qiagen, UK) vortexed and incubated while shaking at 56 ˚C for 1 hr. Samples were again centrifuged 15,000 xg for 15 min (RT) and the supernatant discarded. Pellets were re-suspended in 275µl enzymatic lysis buffer for gram-positive bacteria (20mM TrisHCL (pH 8.0), 2 mM sodium EDTA, 1.2 % Triton X-100, 20 mg/ml lysozyme) and 200 µl 0.1 mm Zirconia/Silica beads (Biospec Products Inc, USA) were added followed by incubation while shaking at 37 °C for 30 min. This was followed by beat beating, 45 sec at 30 rpm, using a TissueLyzer II (Retsch, Germany). To reduce foaming, samples were spun down for 30 sec at 15,000 xg. Then 25 µl proteinase-K and 300 µl Qiagen DNeasy lysis (AL) buffer was added and incubated with gentle shaking at 56 °C for 2 hr followed by bead beating for 45 sec at full speed (30 rpm) and centrifugation at 12,000 xg for 10 min. The supernatant was transferred to a new reaction tube without beads and 100% ethanol added at the ratio of 1 to 2 (ethanol: supernatant). DNA extraction procedure followed the Spin-Column protocol from the Qiagen DNeasy Blood and Tissue kit (Qiagen, UK). DNA was eluted in 50 µl AE buffer and frozen at -20 °C. All samples were diluted 5 times and then only 1/10 of each sample was assayed to avoid PCR inhibition due to possible DNA overload.

Quantitative PCR was performed using an in house system (Thakur, 2012) validated against a MAP DNA /spiked tissue DNA calibration curve using reactions targeting IS*900* with the validated primer set 5'-GGCAAGACCGACGCCAAAGA-3', 5'-GGGTCCGATCAGCCACCAGA-3'. IS*900* was used in preference to FadE5 due to its presence in multiple copies that ensure increased sensitivity and robustness of testing low loads. All samples were run in duplicate as above with denaturation at 95°C, followed by 45 cycles of 95 °C : 30 sec, 68°C : 60 sec. Data analysis was performed using the Rotor-Gene Q Series Software version 1.7 using a calibration set of DNA dilutions from a standard MAP DNA stock included in all runs (efficiency: 89%, R^2: 0.99780). The lower detection limit was determined according to a standard Cq of 33.57 representing a previously estimated sensitivity of approximately 1.7 Geq. Acceptable duplicate variation was set at 1.5 Cq.

In review

Culture

Hexadecylpyridinium chloride (HPC: Sigma, UK) sample decontamination was carried out according to World Organisation for Animal Health (OIE) standard protocols (Edwards, 2007). Briefly, 100 mg of homogenised sample was added to 1.5 ml sterile 0.75% HPC and incubated at 37 °C for 3 hr. Samples were centrifuged at 1,600 xg for 30 min, supernatant discarded, the pellet resuspended in 0.5 ml of ½ strength Muller Hinton (½MH) Broth with 100 µg/ml Vancomycin, 50 µg/ml Amphotericin B, 100 µg/ml Nalidixic acid and incubated overnight at 37 °C with gentle shaking. Tubes were allowed to settle for 30 min and 100 µl inoculum taken from the middle of the suspension.

TiKa-Kic sample decontamination used 100 mg of homogenized sample digested for 5 hours at 37 °C with gentle shaking (200 rpm) in 1 ml CT digest buffer (8.5 mg/ml NaCl, 1 mg/ml $CaCl_2$, 100 mg/ml Collagenase B (Roche, UK) and 100 mg/ml Trypsin). Digests were centrifuged at 14,000 xg for 10 min and the supernatant discarded. The pellet was then re-suspended in 10 ml ½MH broth supplemented with TiKa-Kic (TiKa Diagnostics, UK) and incubated for 24 hr at 37 °C with gentle shaking. Samples were centrifuged 1,600 xg for 30min and the pellet re-suspended in 600 µl sterile PBS.

TiKa14D-7H11 solid culture used Middlebrook 7H11, 10% OADC , 2 mg/ml Mycobactin J (ID-Vet, France), 25 µg/ml Vancomycin, 30 µg/ml Amphotericin B, 25 µg/ml Nalidixic acid, 1 µg/ml TiKa-14D and was inoculated with effectively 1/6 (100µl) of each treated sample. All plates were read from 3 weeks and cfu's confirmed at 10 weeks.

TiKa-MGIT liquid culture used 7ml MGIT tubes with 0.8 ml growth supplement, 2 mg/ml Mycobactin J (ID-Vet, France), 25 µg/ml Vancomycin, 30 µg/ml Amphotericin B , 25 µg/ml Nalidixic acid, 1 µg/ml TiKa-14D and were inoculated with 5/6 (500µl) of each treated sample. Sample loads were calculated by extrapolation using a calibration algorithm from Time to Positivity (TTP measured in days) of each MGIT tube when flagged positive by the Bactec MGIT 320 mycobacterial detection system as determined by the manufacturer. The calibration algorithm cfl/g tissue = $(3x10^{6})^{(-0.347xTTPdays)}$ was determined from a previous spiking experiments of known dilutions of MAP (strain K10) grown in TiKa14D+MGIT media (data not shown). Processing by qPCR for IS*900* was performed in a separate research facility to culture and all results and identities of animal groups were kept blinded until the end of the experiment.

Samples from various tissue sites obtained from each animal at necropsy were processed in parallel to compare standard and TiKa-Kic methods. They were then quantitatively tested in parallel for the presence of either MAP genome equivalents (Geq) by qPCR, colony forming units (cfu) using TiKa14D+7H11 colony counting or extrapolated colony forming load (cfl) by determining initial inoculum load from TTPdays results of TiKa14D+MGIT liquid culture. Samples from one cull set were additionally tested by MAP culture on unmodified 7H11 plates after preparation using a conventional HPC based method (cfu, HPC). Mean individual total counts for each of the methods (cfu/cfl/Geq) were determined by summing the estimated load from each of the 10 samples from each animal.

In review

Statistical analysis

All statistical analyses were performed using Graph Pad Prism v6.01. For all analyses, a *p* value of <0.05 was considered to be statistically significant. The criteria for interpreting the Spearman's rank correlation with p value < 0.001 was > 0.75 = excellent, < 0.40 = poor, 0.40 - 0.75 = good (Fleiss et al., 2003).

RESULTS

Seven blocks of 7 calves (n=49) were purchased at 6 weeks of age and randomly assigned to vaccine or sham-vaccinated groups. Results of individual vaccine efficacy will be reported elsewhere. Two animals were euthanized as a result of causes unrelated to the experiment (1 broken leg, 1 malformed pyloric sphincter) and were excluded. The remainder, comprising 34 vaccinated and 13 sham-vaccinated (Saline) individuals were successfully challenged 3 weeks post vaccination with an oral dose of MAP (total inoculum estimated by qPCR : $1x10^{13}$ Geq) and maintained in appropriate housing for 28 weeks post challenge. Final MAP loads adjusted for sample weight and collated as cfu, cfl or Geq per 100 mg of sample tissue (Supplementary Table 1) showed all animals had at least 70% of samples positive for MAP by at least one method with 3% (16/470) being negative by all three methods. Sample contamination due to direct carry over of viable non-mycobacterial flora was seen in only 1 of 470 sample preparations using the TiKa-Kic with TiKa14D+MGIT liquid culture. No carry over contamination was seen using HPC with 7H11 or TiKa-Kic with TiKa14D+7H11 culture.

Comparing total load estimates of individual vaccinated animals with the sham-vaccinated (saline) control group we were able to identify a subgroup (designated as 'reactor' group) within the vaccinated animal group that had total load means significantly (cfu: p = <0.0001; cfl: p = <0.0002; Geq: p = 0.042) below that of controls by all three methods (Figure 1).

The conventional HPC+7H11 solid method performed the poorest relative to all other methods. Cultures showed an apparently random proportion of large colonies indicative of clumping present in all HPC positive cultures and required a significantly longer period (TiKa-Kic with TiKa14D+7H11 culture 3-4 weeks, HPC 8-10 weeks) to generate visible colonies. Contrastingly, nearly all colonies grown using TiKa-Kic with TiKa14D+7H11 culture were of a regular size and not suggestive of growing from clumps. There was good correlation of load estimates (r = 0.810: p = <0.0001) between Geq from qPCR and cfu from HPC at high sample loads (Figure 2A) but this was shifted from a 1:1 relationship by a mean 189 fold (median 72 fold) difference. In addition the sensitivity of detection (negative cut off) for HPC treated sampling was relatively low with 37% (12/30) positive qPCR values showing no cfu on HPC with 7H11 solid medium even after 12 weeks incubation. In this small sample comparison the TiKa-Kic with TiKa14D+7H11 protocol was markedly superior to HPC with conventional 7H11 solid medium. TiKa-Kic with TiKa14D+7H11 culture provided a mean 29 fold (median 8 fold) increase in cfu values relative to HPC with 7H11 culture (Figure 2B) and markedly improved recovery with MAP being grown from all HPC negatives (range 7-6129 cfu/100mg).

TiKa solid culture showed excellent correlation with qPCR, particularly in mucosal samples (r = 0.963 p = <0.0001) that approached a linear relationship (mean 13 fold, median 1.6 fold differences in count loads). When these data were separated into populations of mucosal and tissue samples the correlation remained excellent but reduced (r = 0.845 p = <0.0001) with fold differences in count loads shifting proportionally towards Geq (mean 117 fold, median 8 fold),

In review

243 suggesting variations influencing cfu values could be tissue specific (Figure 2C). Extension of
244 this analysis correlating qPCR Geq values and TiKa-Kic with TiKa14D+7H11 cfu across all
245 tested animals confirmed this observation (Figure 3). Correlation of Geq and cfu using TiKa-
246 Kic with TiKa14D+7H11 across all (n=282) animal mucosal samples (Figure 3A) was good (r
247 = 0.723, p = <0.0001) with a close linear relationship over a wide range of values (mean 2 fold,
248 median 1 fold). A significant shift was again observed in mean differences between Geq and
249 cfu in non-mucosal tissue samples with mean 19 fold (median 8 fold) for controls that increased
250 significantly to 232 fold (median 57 fold) if the vaccine 'reactor' group was considered
251 separately (Figure 3B). In this experiment, TiKa-Kic with TiKa14D+7H11 culture was more
252 sensitive than qPCR with 28% (79/282) samples cfu positive / qPCR Geq negative (range 2-
253 156 cfu /100 mg tissue). In contrast only 1% (3/282) of mucosal samples were cfu negative /
254 qPCR Geq positive (range 36-209 Geq/100mg tissue).

255 Correlations with qPCR and TiKa-Kic with TiKa14D+MGIT liquid culture had similar
256 outcomes with near linear correlations in mucosal samples (Supplementary Figure A) and a
257 significant shift in mean differences of 107 fold (median 53 fold) in 'reactor' animals relative
258 to qPCR. TiKa-Kic with TiKa14D+MGIT liquid culture was also more sensitive than qPCR
259 with 28% (79/282) samples cfl positive / qPCR Geq negative (range 2-156 cfl /100 mg tissue)
260 and only 1% (3/282) of mucosal samples were cfl negative / qPCR Geq positive (range 36-209
261 Geq /100 mg tissue).

262

263 **DISCUSSION**

264 The slow nature of MAP growth and its consequent sample turnaround time with conventional
265 culture has promoted the development of highly sensitive molecular methods as an attractive
266 rapid alternative. However molecular quantification of genome equivalents as a measure does
267 not provide any indication of load viability. Previous comparative studies of qPCR and
268 conventional culture have demonstrated good correlation in high sample loads present in faeces
269 (Douarre et al., 2010; Mita et al., 2016) but the inability of conventional culture methods to
270 accurately culture low loads of viable organisms from clinical samples introduces problems.
271 Culture sensitivity for MAP has only ever been as good as 2-3 $\log_{10}$ (Ricchi et al., 2016), thus
272 at low loads correlations to DNA presence are difficult to obtain and the true relationship
273 between genome equivalent values and the demonstrable viable MAP count in these samples
274 remains uncertain (Kralik et al., 2012; Plain et al., 2015). The underlying reasons for these
275 discrepancies are probably multi-variant. DNA detection from difficult samples such as faeces
276 are often significantly influenced by carry-through of amplification enzyme inhibitors and the
277 necessity for multiple steps in sample processing that can introduce error (Timms et al., 2015).
278 Furthermore, the need to decontaminate samples of commensal bacterial and fungal flora
279 introduces MAP exposure during sample preparation to chemicals and antibiotics that can
280 inhibit and in some cases kill large proportions of the viable load (Gumber and Whittington,
281 2007; Kralik et al., 2014). These variables make it difficult to define the number of samples
282 and quantity of any one sample which should be tested to gain significant confidence when
283 assessing true test negativity.

284 In this study we have used samples available from a vaccine-challenge experiment to evaluate
285 the Tika culture system which uses a novel sample preparation protocol (TiKa-Kic) and
286 supplemented growth media (TiKa+7H11, TiKa+MGIT). The sample preparation method

In review

287 differs from conventional protocols in not requiring harsh chemical treatment of samples such
288 as HPC, sodium hydroxide and oxalic acid to remove contaminating flora. The TiKa-Kic
289 killing cocktail has no influence on mycobacterial growth or viability but is effective against a
290 wide range of other bacterial and fungal genera. When followed by growth in conventional
291 MAP media (either liquid or solid) supplemented with a growth enhancer (TiKa14D) there is
292 stimulation of MAP growth and suppression of both MAP aggregation/clumping and entry into
293 lag phase. Parallel processing of samples from experimentally challenged calves comparing
294 TiKa with a standard HPC sample protocol showed a mean 184 fold greater growth of MAP
295 load with colonies appearing 3-4 weeks before the majority of colonies detected using HPC
296 treated samples. In addition 30% of qPCR positive processed samples when processed with
297 conventional HPC treatment, produced no visible recovery after 8 -10 weeks incubation whilst
298 TiKa-Kic treatment allowed growth of regular sized colonies in each of these samples.
299 Parallel processing of mucosal tissue samples from experimentally infected calves showed that
300 TiKa culture gave excellent correlations at medium and high MAP loads with genome
301 equivalent (Geq) estimates derived using a qPCR with a dynamic range cut off of 1.7 Geq. A
302 major finding of this study showed that TiKa culture was the most sensitive test with 22% of
303 cfu positive TiKa treated samples from control animals being negative in qPCR (mean = 455
304 cfu : range 14-1564 cfu). This difference could be explained to some extent by the specific
305 sample volume used for qPCR which was required to be 20 fold less than for TiKa sampling
306 to ensure avoidance of carry over inhibitors. These results suggest TiKa culture was able to
307 consistently recover and grow colonies from a significant majority of the MAP load within
308 mucosal tissue and that this represents a 2 $\log_{10}$ improvement over any existing culture
309 protocol. Subsequent testing using this qPCR method should consider raising the sample
310 volume if possible.

In review

311 Further analysis of the data was able to discern that fold differences between MAP genome
312 equivalent loads (qPCR) and MAP viable loads (cfu) in lymph node tissues was significantly
313 and consistently different to that of mucosal samples. Correlations of cfu and Geq values from
314 MAP infected control animals remained linear but produced a mean 19 fold (median 8 fold)
315 decrease in culturable MAP load of lymphatic tissue compared to mucosal tissue. There was
316 no evidence of increases in clumping in these samples and the correlation appeared good over
317 the whole range of loads suggesting that this was not a technical phenomenon. Similar shifts
318 were observed in both solid and liquid TiKa supplemented media. We surmise that lymphatic
319 tissue was evoking a significant effect on the viability of this proportion of MAP in all animals
320 regardless of vaccination status. Importantly this was not the case in mucosal tissue suggesting
321 that this effect was tissue specific. Interestingly, a sub group of the animals (referred to here as
322 'reactors') that had been MAP-vaccinated prior to MAP challenge and had responded by
323 decreasing the MAP load in both mucosal and lymphatic tissue significantly below any of the
324 sham-vaccinated controls showed the largest effect in this regard. Vaccine 'reactors' had a
325 mean 100 fold greater reduction in fold differences between Geq and cfu than seen in mucosal
326 tissue from the same animals and 10 fold greater reduction than equivalent tissue from sham-
327 vaccinated controls. This data suggests that at the selected time point (7 months post challenge)
328 MAP vaccine 'reactors' were harbouring up to 3 $\log_{10}$ greater proportion of dead or possibly
329 non-culturable/viable 'dormant' MAP in their lymphatic tissue. Why this population should be
330 absent from mucosal tissue requires more directed studies including detailing the predominant
331 metabolic state and degree of viability of MAP in active lymphatic tissue. We hypothesise that
332 vaccine 'reactors' are generating more active MAP killing mechanisms and the increased
333 residual DNA presence in lymphatic tissue is a result of the detection of killed MAP which

334 unlike the mucosal compartment have not yet been fully processed and translocated for
335 excretion.

336

337

338 **CONCLUSION**

339 TiKa culture provides the most efficient and rapid method of culturing MAP so far described.
340 It validates the use of qPCR for rapid determination of viable MAP load in mucosal tissue and
341 when applied together with qPCR could offer the possibility of a novel method to monitor
342 vaccine efficacy. Importantly this study shows that qPCR is not an accurate method of
343 quantifying viable MAP load in lymphatic tissue as this varies widely within individuals with
344 diverse immunologically reactive status. Future studies are required to evaluate the utility of
345 TiKa culture and determine the true proportion of MAP viability in these samples.

346

347 **AUTHOR CONTRIBUTIONS**

348 GJ conceived and designed the experiments. TB co-ordinated culture experiments, analysed
349 data and wrote the paper in collaboration with all authors. GJ, JSG, SBH, HM, MRS and TM
350 performed experiments and analysed data. KH, PLP, SH contributed reagents and materials.

351

In review

352 **ACKNOWLEDGEMENTS**

353 Jeanne Toft Jacobsen and Lien Thi Minh Nguyen are acknowledged for technical assistance.

354

355 **ETHICS**

356 All animal procedures were approved and controlled by the Danish National Experiments
357 Inspectorate.

358

359 **CONFLICT OF INTEREST STATEMENT**

360 T. J. Bull and K. Hilpert declare their affiliation with TiKa Diagnostics Ltd. The company did
361 not influence the design, conduction, interpretation or evaluation of this study.

362

363 **REFERENCES**

364

365 Botsaris, G., Swift, B.M., Slana, I., Liapi, M., Christodoulou, M., Hatzitofi, M., et al. (2016).
366 Detection of viable *Mycobacterium avium* subspecies *paratuberculosis* in powdered
367 infant formula by phage-PCR and confirmed by culture. *Int J Food Microbiol* 216**,** 91-
368 94.

Bull, T.J., McMinn, E.J., Sidi-Boumedine, K., Skull, A., Durkin, D., Neild, P., et al. (2003). Detection and verification of *Mycobacterium avium* subsp. *paratuberculosis* in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. *J.Clin.Microbiol.* 41(7)**,** 2915-2923.

Douarre, P.E., Cashman, W., Buckley, J., Coffey, A., and O'Mahony, J.M. (2010). Isolation and detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from cattle in Ireland using both traditional culture and molecular based methods. *Gut Pathog* 2(1)**,** 11.

Edwards, S. (2007). OIE standards for vaccines and future trends. *Rev Sci Tech* 26(2)**,** 373-378.

Elguezabal, N., Bastida, F., Sevilla, I.A., Gonzalez, N., Molina, E., Garrido, J.M., et al. (2011). Estimation of *Mycobacterium avium* subsp. *paratuberculosis* growth parameters: strain characterization and comparison of methods. *Appl Environ Microbiol* 77(24)**,** 8615-8624.

Fleiss, J.L., Levin, B.A., and Paik, M.C. (2003). *Statistical methods for rates and proportions.* Hoboken, N.J.: J. Wiley.

Galiero, A., Fratini, F., Mataragka, A., Turchi, B., Nuvoloni, R., Ikonomopoulos, J., et al. (2016). Detection of *Mycobacterium avium* subsp. *paratuberculosis* in cheeses from small ruminants in Tuscany. *Int J Food Microbiol* 217**,** 195-199.

Gitlin, L., Borody, T.J., Chamberlin, W., and Campbell, J. (2012). *Mycobacterium avium* ss *paratuberculosis*-associated Diseases: Piecing the Crohn's Puzzle Together. *J.Clin.Gastroenterol.* 46(8)**,** 649-655.

Grant, I.R., and Rowe, M.T. (2004). Effect of chemical decontamination and refrigerated storage on the isolation of *Mycobacterium avium* subsp. *paratuberculosis* from heat-treated milk. *Lett.Appl.Microbiol.* 38(4)**,** 283-288.

Gumber, S., and Whittington, R.J. (2007). Comparison of BACTEC 460 and MGIT 960 systems for the culture of *Mycobacterium avium* subsp. *paratuberculosis* S strain and observations on the effect of inclusion of ampicillin in culture media to reduce contamination. *Vet Microbiol* 119(1)**,** 42-52.

Harris, N.B., and Barletta, R.G. (2001). *Mycobacterium avium* subsp. *paratuberculosis* in Veterinary Medicine. *Clin.Microbiol.Rev.* 14(3)**,** 489-512.

Hines, M.E., 2nd, Stabel, J.R., Sweeney, R.W., Griffin, F., Talaat, A.M., Bakker, D., et al. (2007). Experimental challenge models for Johne's disease: a review and proposed international guidelines. *Veterinary microbiology* 122(3-4)**,** 197-222.

Kalis, C.H., Hesselink, J.W., Barkema, H.W., and Collins, M.T. (2001). Use of long-term vaccination with a killed vaccine to prevent fecal shedding of *Mycobacterium avium* subsp *paratuberculosis* in dairy herds. *Am.J.Vet.Res.* 62(2)**,** 270-274.

Kralik, P., Babak, V., and Dziedzinska, R. (2014). Repeated cycles of chemical and physical disinfection and their influence on *Mycobacterium avium* subsp. *paratuberculosis* viability measured by propidium monoazide F57 quantitative real time PCR. *Vet J* 201(3)**,** 359-364.

Kralik, P., Beran, V., and Pavlik, I. (2012). Enumeration of *Mycobacterium avium* subsp. *paratuberculosis* by quantitative real-time PCR, culture on solid media and optical densitometry. *BMC Res Notes* 5**,** 114.

McAloon, C.G., Whyte, P., More, S.J., Green, M.J., O'Grady, L., Garcia, A., et al. (2016). The effect of paratuberculosis on milk yield--A systematic review and meta-analysis. *J Dairy Sci* 99(2)**,** 1449-1460.

Mita, A., Mori, Y., Nakagawa, T., Tasaki, T., Utiyama, K., and Mori, H. (2016). Comparison of fecal pooling methods and DNA extraction kits for the detection of *Mycobacterium avium* subspecies *paratuberculosis*. *Microbiologyopen* 5(1)**,** 134-142.

In review

419 Nazareth, N., Magro, F., Appelberg, R., Silva, J., Gracio, D., Coelho, R., et al. (2015).
420 Increased viability but decreased culturability of *Mycobacterium avium* subsp.
421 *paratuberculosis* in macrophages from inflammatory bowel disease patients under
422 Infliximab treatment. *Med Microbiol Immunol* 204(6)**,** 647-656.
423 Park, K.T., Allen, A.J., and Davis, W.C. (2014). Development of a novel DNA extraction
424 method for identification and quantification of *Mycobacterium avium* subsp.
425 *paratuberculosis* from tissue samples by real-time PCR. *J Microbiol Methods* 99**,** 58-
426 65.
427 Plain, K.M., Marsh, I.B., Waldron, A.M., Galea, F., Whittington, A.M., Saunders, V.F., et al.
428 (2014). High-throughput direct fecal PCR assay for detection of *Mycobacterium avium*
429 subsp. *paratuberculosis* in sheep and cattle. *J Clin Microbiol* 52(3)**,** 745-757.
430 Plain, K.M., Waldron, A.M., Begg, D.J., de Silva, K., Purdie, A.C., and Whittington, R.J.
431 (2015). Efficient, validated method for detection of mycobacterial growth in liquid
432 culture media by use of bead beating, magnetic-particle-based nucleic acid isolation,
433 and quantitative PCR. *J Clin Microbiol* 53(4)**,** 1121-1128.
434 Ricchi, M., Savi, R., Bolzoni, L., Pongolini, S., Grant, I.R., De Cicco, C., et al. (2016).
435 Estimation of *Mycobacterium avium* subsp. *paratuberculosis* load in raw bulk tank milk
436 in Emilia-Romagna Region (Italy) by qPCR. *Microbiologyopen* 5(4)**,** 551-559.
437 Thakur, A. (2012). *Development of a multi-stage vaccine against paratuberculosis in cattle.*
438 PhD, Technical University of Denmark, Copenhagen
439 Timms, V.J., Daskalopoulos, G., Mitchell, H.M., and Neilan, B.A. (2016). The Association of
440 *Mycobacterium avium* subsp. *paratuberculosis* with Inflammatory Bowel Disease.
441 *PLoS One* 11(2)**,** e0148731.
442 Timms, V.J., Mitchell, H.M., and Neilan, B.A. (2015). Optimisation of DNA extraction and
443 validation of PCR assays to detect *Mycobacterium avium* subsp. *paratuberculosis*. *J*
444 *Microbiol Methods* 112**,** 99-103.
445 Whittington, R.J. (2009). Factors affecting isolation and identification of *Mycobacterium*
446 *avium* subsp. *paratuberculosis* from fecal and tissue samples in a liquid culture system.
447 *J Clin Microbiol* 47(3)**,** 614-622.

448

449

In review

LEGENDS

FIGURE 1: Distribution plots of total MAP loads in different methods. Plots derived by summing loads from 10 samples for each animal determined from the same set of samples (n=470) by three separate methods **(A)** cfu using TiKa-Kic/Tika14D+7H11 method (p=<0.0001) **(B)** cfu using TiKa-Kic/Tika14D+MGIT method (p=<0.0002) and **(C)** Geq using qPCR (p=<0.042) on control and vaccinated groups of calves. There was no statistical significance between Saline and Non-reactor groups in all methods.

FIGURE 2: Correlation plots of MAP loads in individual samples from mucosal and non-mucosal tissues. These plots were estimated by three methods **(A)** cfu by HPC with qPCR at median 72 fold, mean 189 fold and Spearman r at 0.810; **(B)** cfu by HPC with cfu by TiKa-Kic/Tika14D+7H11 at median 8 fold, mean 29 fold and Spearman r at 0.723 and **(C)** cfu by TiKa-Kic/Tika14D+MGIT with qPCR at median 1.6 fold, mean 13 fold and Spearman r at 0.963 for mucosal samples and at median 8 fold, mean 117 fold and Spearman r at 0.845 for non-mucosal samples (qPCR = Geq). Dotted lines represent medians.

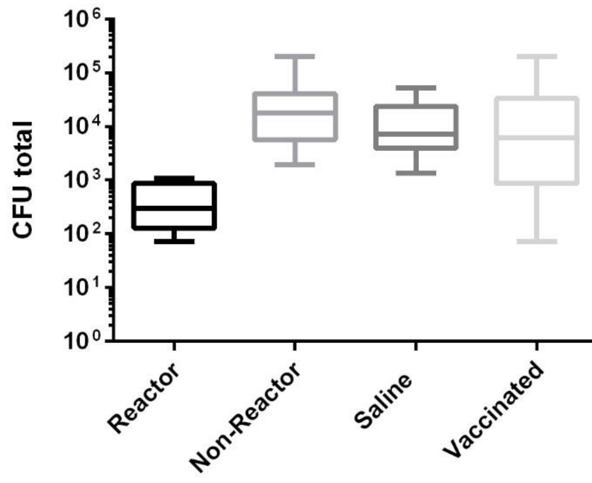
FIGURE 3: Correlation plots of MAP loads comparing TiKa14D+7H11 and qPCR. Individual samples estimated as cfu by TiKa-Kic/Tika14D+7H11 and Geq by qPCR for **(A)** mucosal having control (median 1 fold, mean 2 fold), vaccinated non-reactor (median 2 fold, mean 2 fold) and vaccinated reactor (median 4 fold, mean 13 fold) groups of calves and **(B)** non-mucosal tissues also having control (median 8 fold, mean 19 fold), vaccinated non-reactor (median 8 fold, mean 29 fold) and vaccinated reactor (median 60 fold, mean 190 fold) groups of calves. Dotted lines represent medians. Spearman r for each was 0.723 and 0.717 respectively.

In review

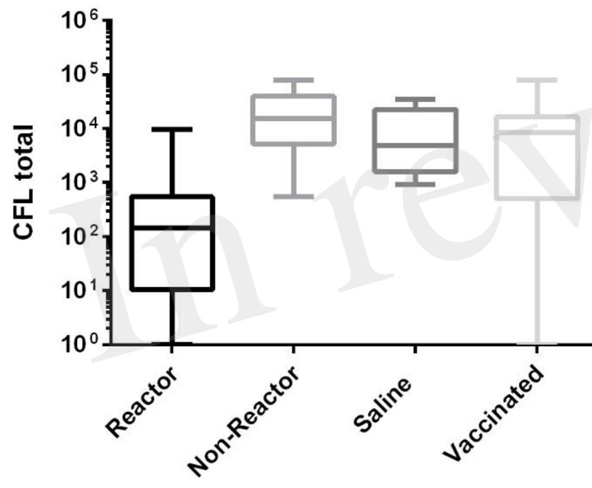
SUPPLEMENTARY FIGURE A: Correlation plots of MAP loads comparing TiKa-MGIT and qPCR. Individual samples estimated as cfu by TiKa-Kic/Tika14D+MGIT and Geq by qPCR for **(A)** mucosal having control (median 1 fold, mean 1 fold), vaccinated non-reactor (median 1 fold, mean 2 fold) and vaccinated reactor (median 1 fold, mean 5 fold) groups of calves and **(B)** non-mucosal tissues also having control (median 30 fold, mean 61 fold), vaccinated non-reactor (median 21 fold, mean 39 fold) and vaccinated reactor (median 53 fold, mean 107 fold) groups of calves. Dotted lines represent medians. Spearman r for each was 0.744 and 0.681 respectively.

FIGURE 1

A

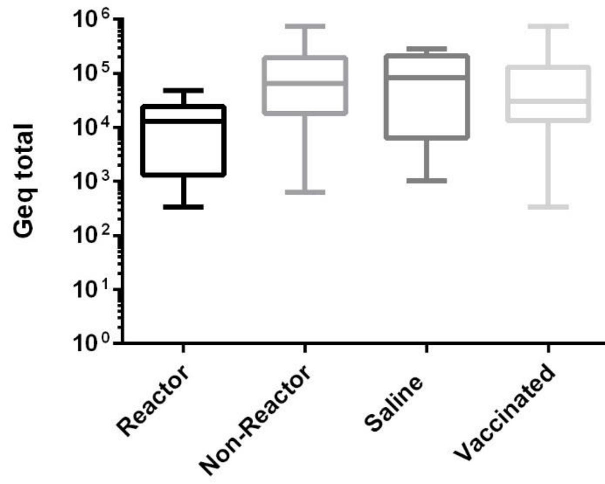


B



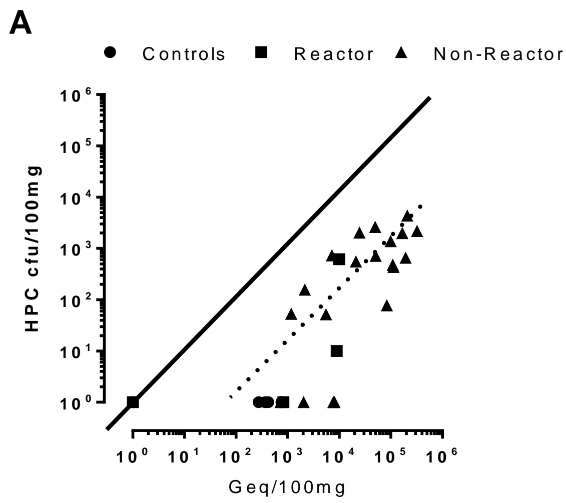
In review

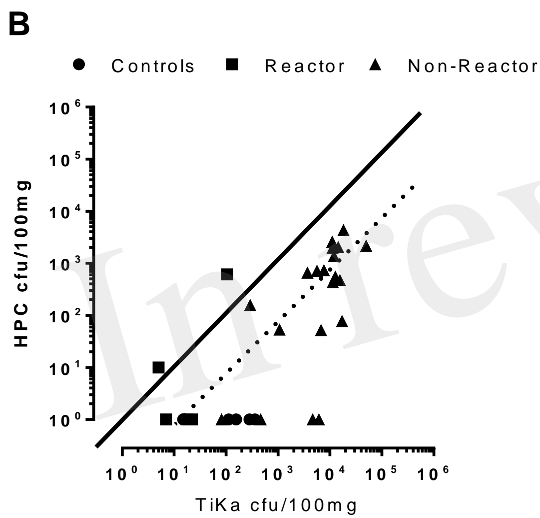
C

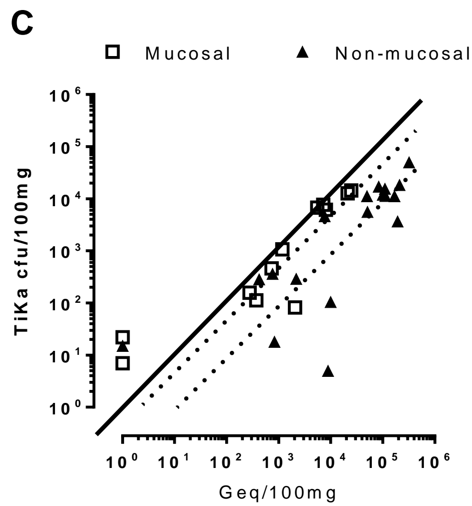


481

482 FIGURE 2



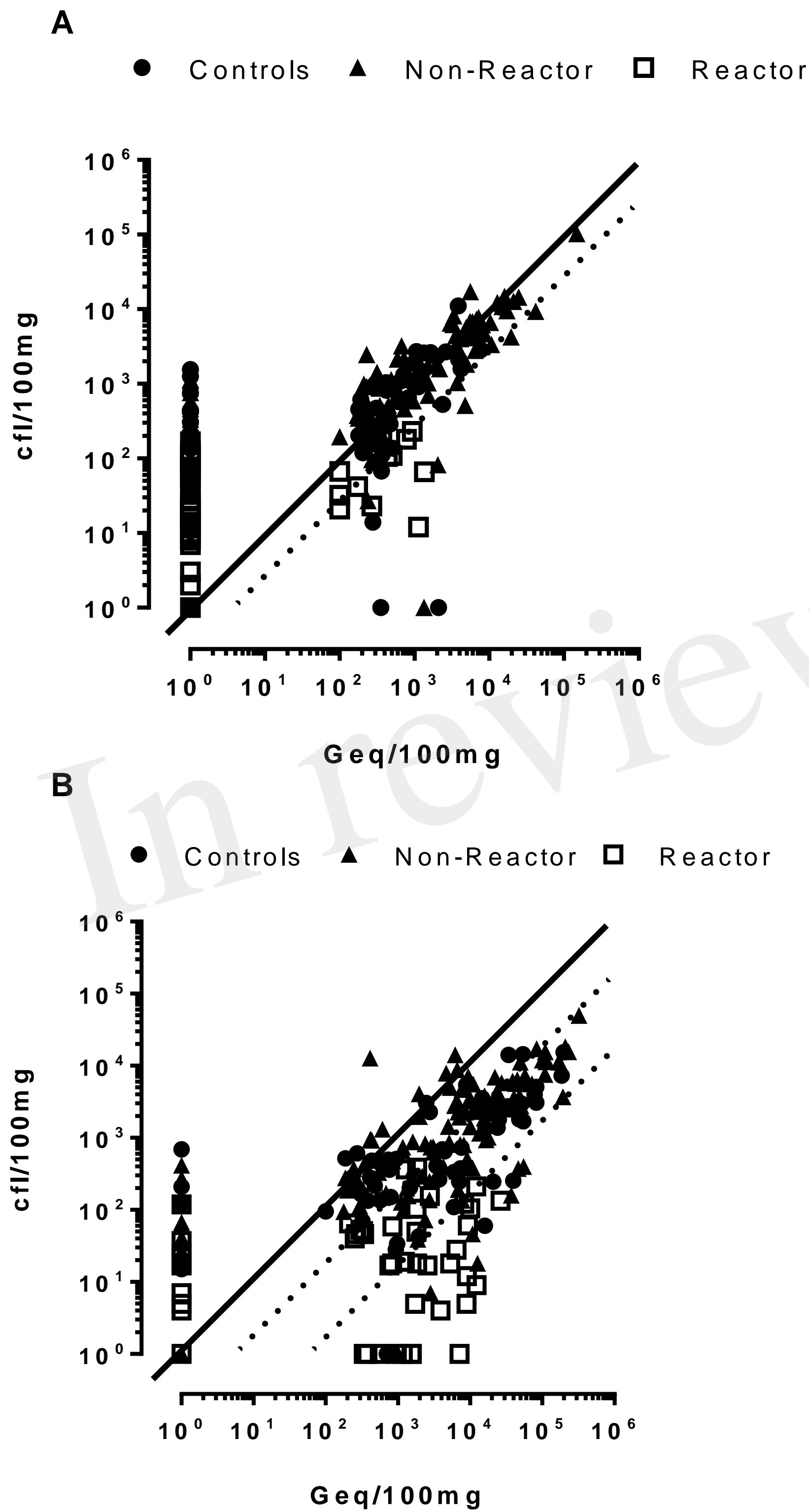




483

In review

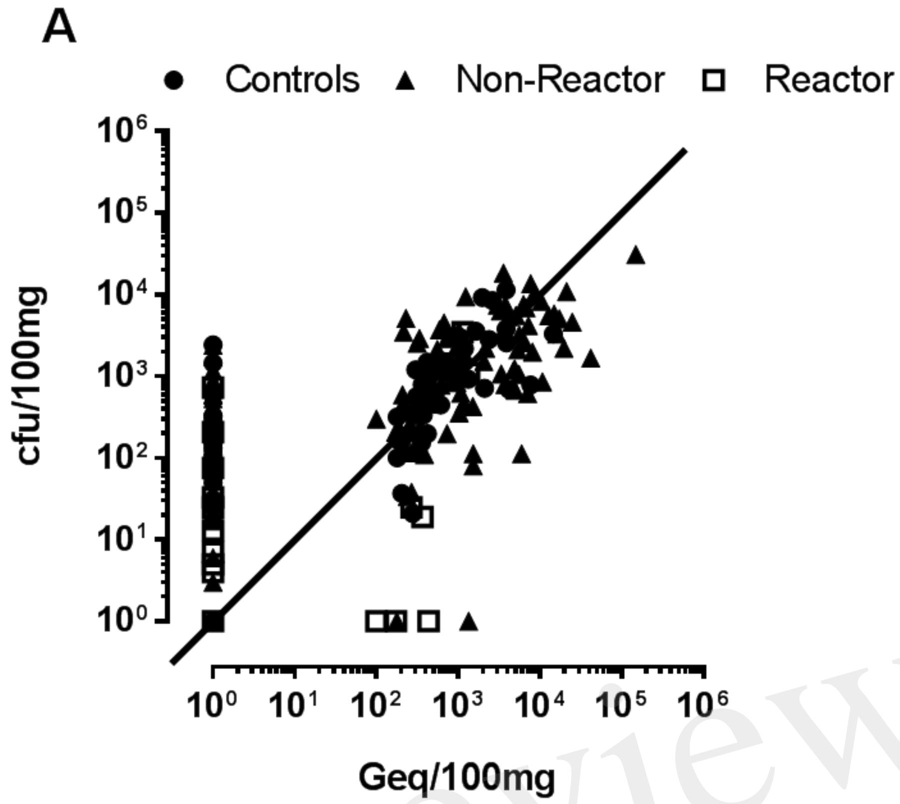
484 FIGURE 3

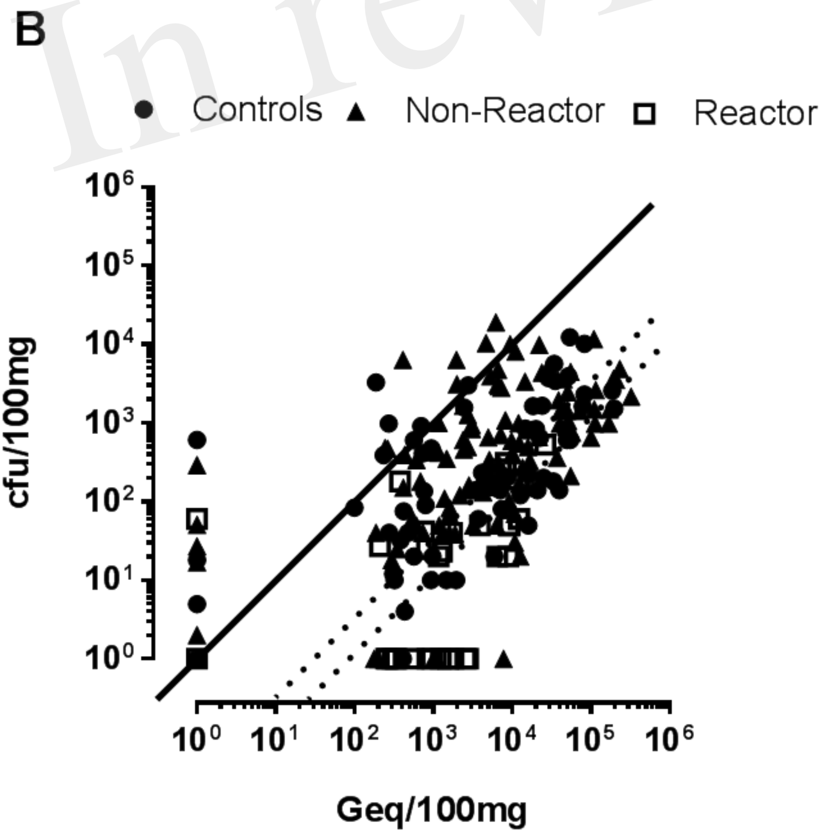


In review

485

486 SUPPLEMENTARY FIGURE A

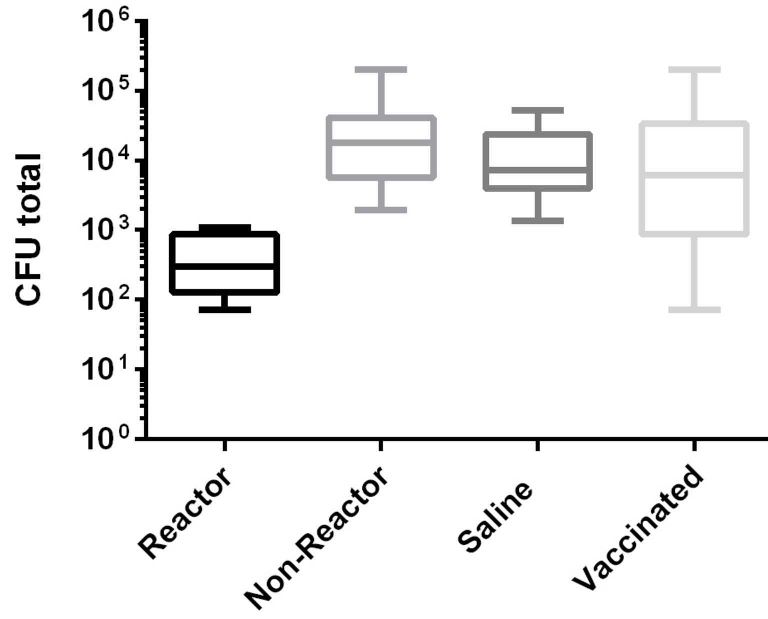


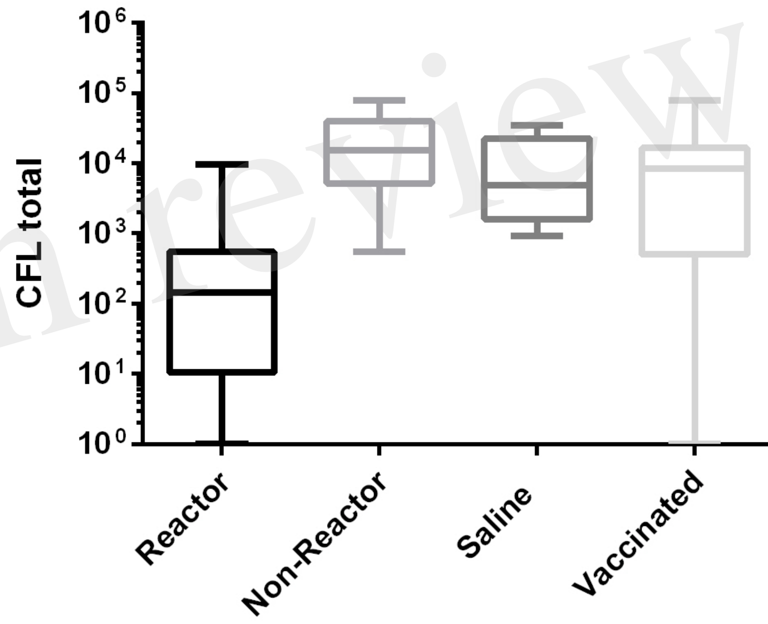


487

In review

Figure 1.JPEG





In review

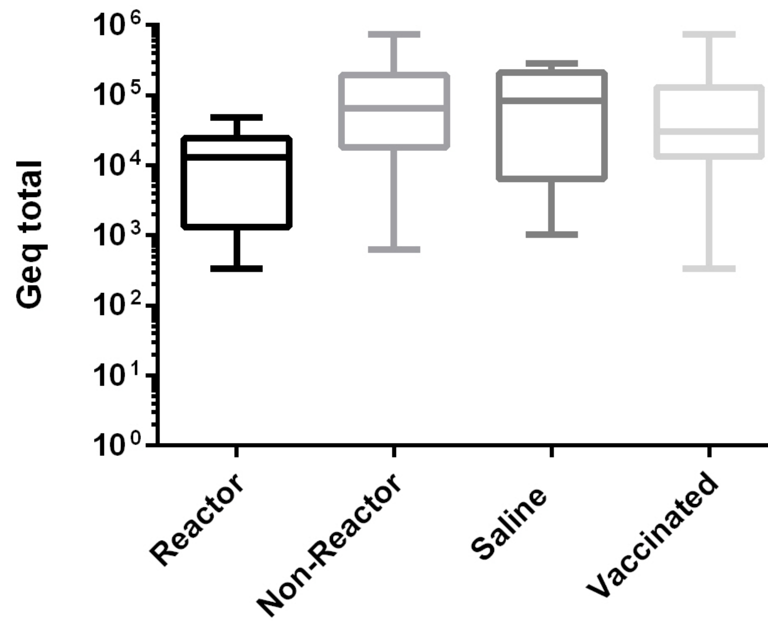
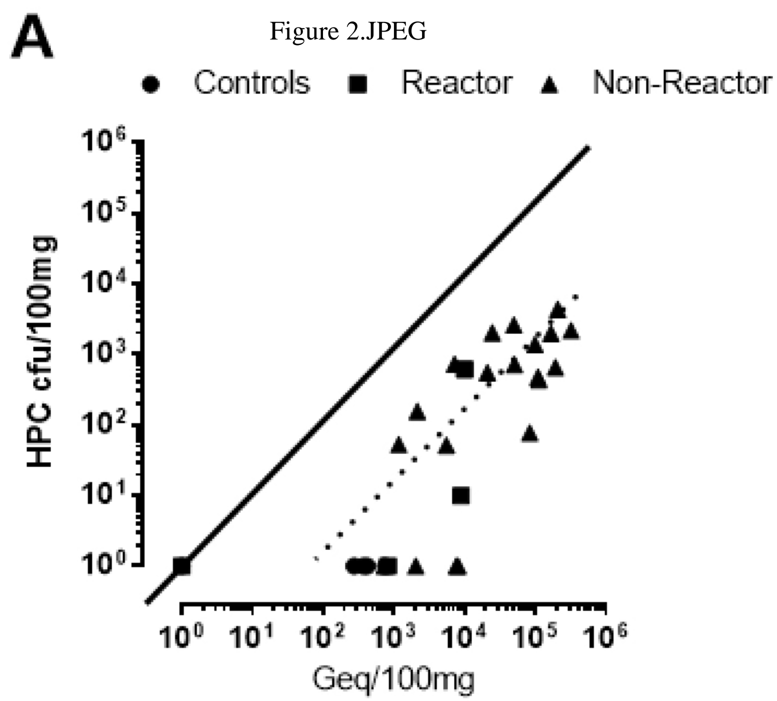
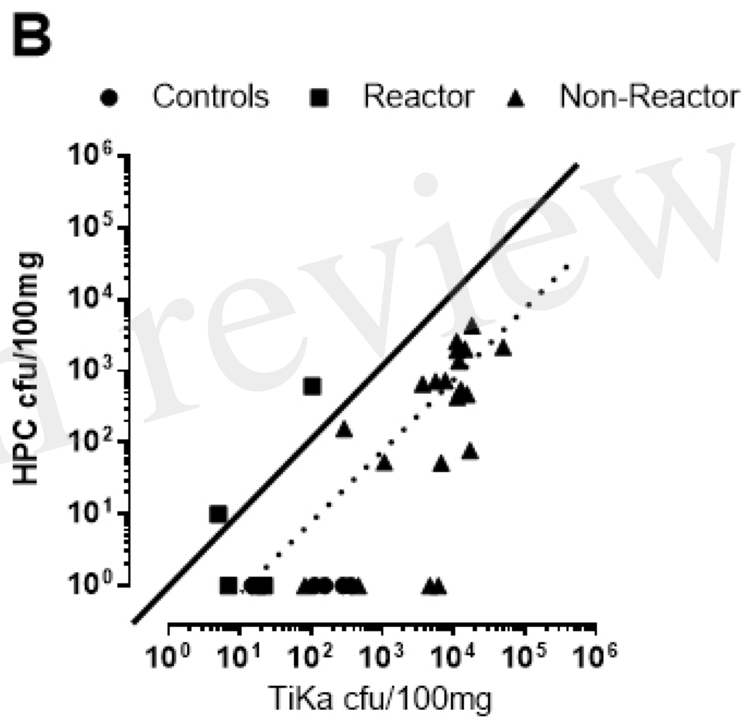
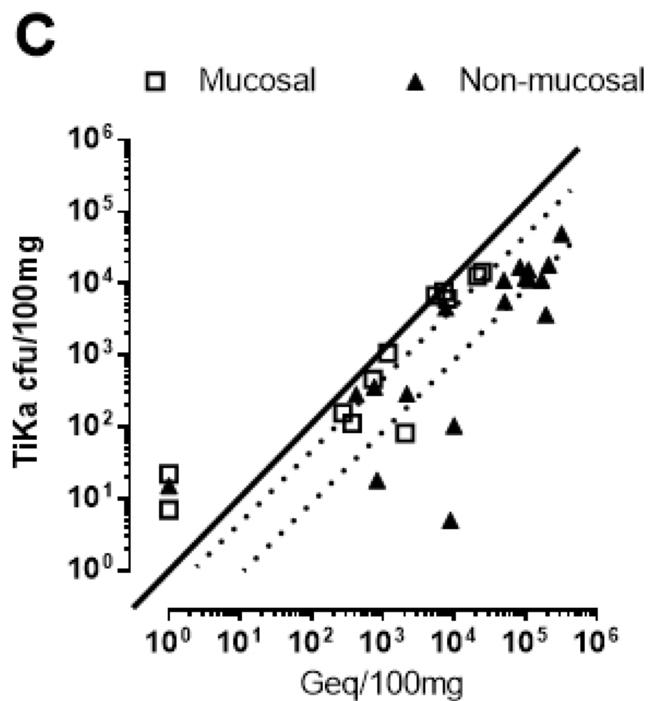


Figure 2.JPEG

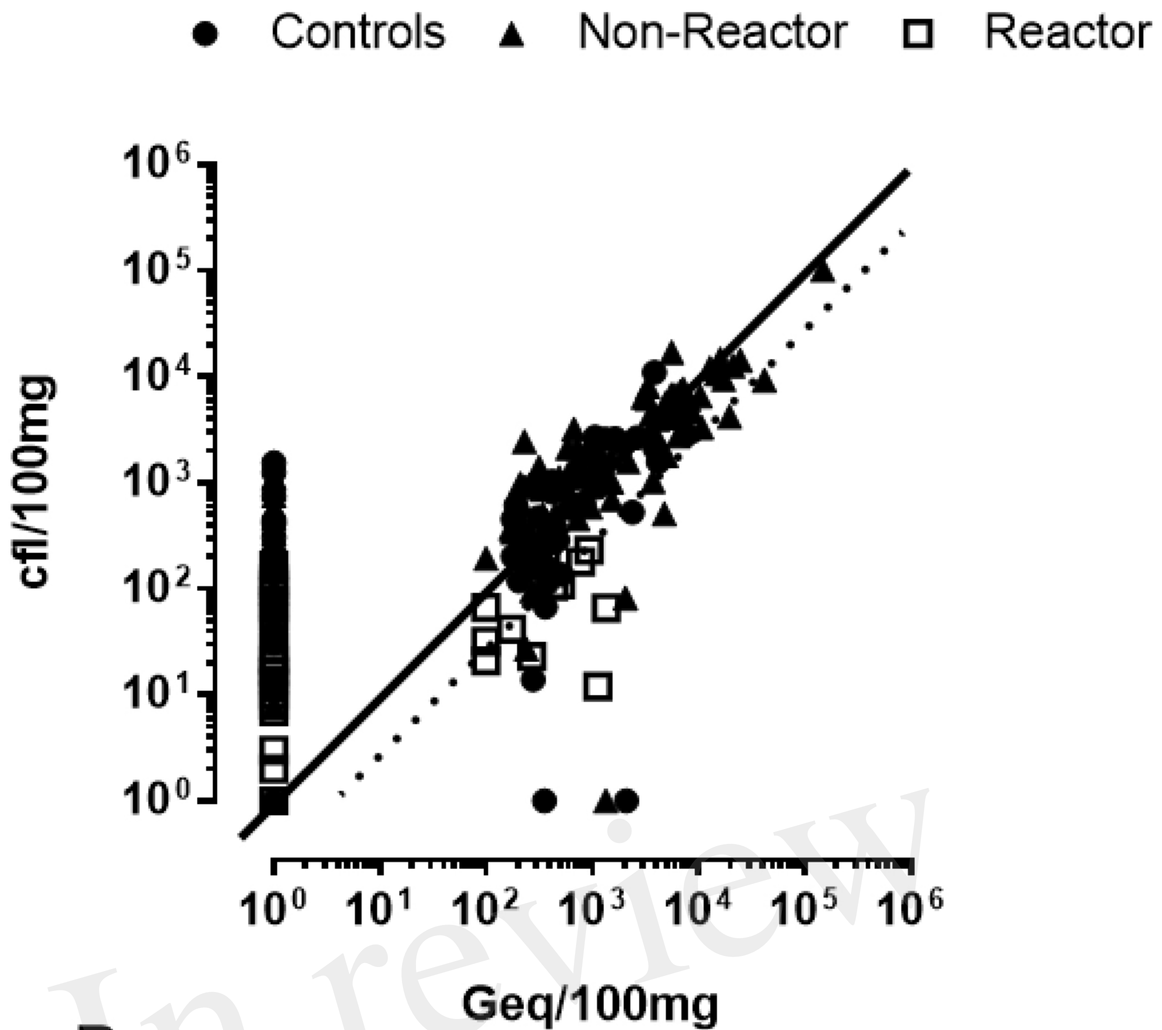






In review

Figure 3.JPEG



In review