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RESEARCH ARTICLE

A qPCR assay for *Bordetella pertussis* cells that enumerates both live and dead bacteria

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Abstract

Bordetella pertussis is the causative agent of whooping cough, commonly referred to as pertussis. Although the incidence of pertussis was reduced through vaccination, during the last thirty years it has returned to high levels in a number of countries. This resurgence has been linked to the switch from the use of whole-cell to acellular vaccines. Protection afforded by acellular vaccines appears to be short-lived compared to that afforded by whole cell vaccines. In order to inform future vaccine improvement by identifying immune correlates of protection, a human challenge model of *B. pertussis* colonisation has been developed. Accurate measurement of colonisation status in this model has required development of a qPCR-based assay to enumerate *B. pertussis* in samples that distinguishes between viable and dead bacteria. Here we report the development of this assay and its performance in the quantification of *B. pertussis* from human challenge model samples. This assay has future utility in diagnostic labs and in research where a quantitative measure of both *B. pertussis* number and viability is required.

Introduction

Whooping cough, or pertussis, is a highly contagious respiratory tract infection of humans caused by the gram-negative coccobacillus *Bordetella pertussis*. Clinical manifestations of pertussis depend on age and immune status of the host and include a low-grade fever, cyanosis, and paroxysmal cough accompanied by a high-pitched "whoop" [1]. Infants aged less than 1 year old present the highest incidence of pertussis and are also at the greatest risk of severe disease and death [2].

The introduction of vaccination in the early 1950s significantly reduced the incidence of pertussis in developed nations, however the number of reports of pertussis has been

progressively increasing over the last thirty years [3]. For example, in the UK, Public Health England has reported a greater than ten-fold increase in pertussis cases over the eight-year period of 2005–2013 [4]. This rise has been echoed in other countries including Australia, the Netherlands, and the US [5–10].

The reason for this resurgence is not certain, however it has been strongly linked to the switch from using whole-cell vaccines (WCVs) to using acellular vaccines (ACVs). ACV-induced immunity appears to wane more quickly than WCV-induced immunity. In baboons, compared to WCV-induced immunity, ACV-induced immunity protects from disease, but does not prevent colonisation by *B. pertussis* or prevent transmission of the bacteria to other hosts [11,12]. Asymptomatic transmission from colonised carriers to naive contacts could be contributing to the resurgence in the US and UK [13]. In many industrialised countries using ACVs (Denmark, Norway, Netherlands, Finland, Sweden, the United Kingdom, United States of America, Australia, France, and Japan) there has been a dramatic increase in the isolation of *B. pertussis* deficient for the production of the ACV-vaccine antigen pertactin [14–18]. In ACV-immunised hosts pertactin-deficient *B. pertussis* may have a fitness advantage over pertactin-producing isolates, raising concern that the use of ACVs is selecting for vaccine escape strains of *B. pertussis* [19]. These issues have highlighted the need to better understand the detailed differences between WCV and ACV induced immune responses and the immune response to infection, and to identify biomarkers of protective immunity to *B. pertussis* infection. This would aid the evaluation of the efficacy of future *B. pertussis* vaccines that might be needed to combat *B. pertussis* resurgence. To this end, a human challenge model of *B. pertussis* colonisation has been developed as part of the EU-funded PERISCOPE Project [20,21]. In this model it is necessary to be able to monitor the colonisation status of participants at frequent intervals. Current detection methods for *B. pertussis* include culture from nasopharyngeal swabs or other nasopharyngeal samples. However, *B. pertussis* is slow-growing and takes several days to produce visible growth on laboratory agar. A more rapid method would improve safety for human challenge model volunteers. Real-time PCR detection (qPCR) of *B. pertussis* DNA provides identification of *B. pertussis* within hours. Use of the multicopy insertion sequence IS481 as a target makes this assay much more sensitive for the diagnosis of *B. pertussis* infection than culture [22]. However, traditional qPCR assays cannot distinguish between viable and dead bacteria, which is essential to determine whether participants are actively colonised. Enumerating viable bacteria is especially important in the prevention of pertussis and associated symptoms during the challenge, as well as transmission to others post-challenge by ensuring the efficacy of treatment.

Here we report the modification of a standard qPCR assay used for laboratory diagnosis of *B. pertussis*, through treatment of samples with propidium monoazide (PMA) that inhibits PCR-mediated amplification of DNA from dead cells and allows distinguishing of viable from dead cells [23–27]. The use of PMA involves an initial incubation of samples with PMA in darkness, during which it diffuses into dead cells, followed by light activation of PMA that permanently modifies the genomic DNA (gDNA) of dead cells, preventing it from acting as a template in PCR. The optimisation of this assay and its use to enumerate viable and dead *B. pertussis* from human challenge model samples is described. In addition, this assay has wider uses in diagnostic and other research settings where a quantitative measure of viable *B. pertussis* number is required.

Materials and methods

Bacterial strains and culture conditions

B. pertussis strain B1917 is a wild-type strain considered representative of currently circulating *B. pertussis* [28]. The streptomycin-resistant *B. pertussis* strain BP536 was derived from

Tohama 1 [29]. *B. pertussis* strains UK48 and UK71 can both be identified with European Nucleotide Archive Accession numbers: ERS176875 and ERS227772, respectively. *B. pertussis* strain B204 (B1878) and B184 (B2973) were both derived from the Netherlands and can be located with the following NCBI Genbank accession numbers: NZ_CSNV00000000 and NZ_CSRZ00000000, respectively. All strains were cultured on charcoal agar (ThermoFisher Scientific, Oxoid™, Basingstoke, UK) at 37˚C for 3 days for routine culture.

The preparation of heat-killed bacterial cell suspensions

Plate-grown B1917 were resuspended in PBS (ThermoFisher Scientific, Oxoid™, Basingstoke, UK) to an OD_{600} = 1.0 (approximately 10^9 cfu/ml). To optimise heat killing, 1 ml aliquots were heat-killed at 80˚C for 1, 3 and 6 minutes in a pre-heated heat block. Aliquots were placed on ice immediately after incubation. Bacterial death was confirmed by the absence of growth after streaking 10 µl of suspension onto charcoal agar plates and incubating at 37˚C for 5 days. To ascertain the integrity of heat-killed cells, samples were subjected to flow cytometry (FACS-CantoII, BD UK Ltd, Wokingham, U.K.). A detergent-lysed sample acted as a positive control for lysis and a sample containing live cells was a positive control for cell integrity.

The preparation of THP-1 cells

THP-1 (ATCCⓇ TIB-202™) cells were maintained in RPMI 1640 medium (ThermoFisher Scientific, Gibco™, Loughborough, UK), fetal bovine serum (10%) (ThermoFisher Scientific, Gibco™, Loughborough, UK), 1% streptomycin, penicillin and glutamine (ThermoFisher Scientific, Loughborough, UK) as per standard methods. Heat-killed THP-1 cells were prepared by incubating cell suspensions at 10^5 cells/ml at 80˚C for 6 minutes in a pre-heated heat block.

Optimisation of PMA treatment conditions

PMA Dye, 20 mM in H_2O (Cambridge BioSciences, Cambridge, UK), was stored at -20˚C in the dark, thawed on ice and added to 2 ml clear centrifuge tubes containing 200 µl of cell suspensions to a final concentration of 20 µM, 30 µM, or 50 µM. PMA-free samples served as controls for each condition tested. Tubes were covered with aluminium foil and shaken on an orbital shaker for 5, 10, 20, 30 or 70 minutes. Samples were then exposed to light using the PMA-Lite LED Photolysis Device (Cambridge BioSciences, Cambridge, UK) for 5, 10, 20, 30 or 40 minutes. Samples were pelleted using the Heraeus Pico 17 Centrifuge at 2000xg (ThermoFisher Scientific, Loughborough, UK) for 10 minutes at room temperature prior to DNA isolation. Non-PMA treated controls allow for the total number of *B. pertussis* cells to be enumerated. The number of viable *B. pertussis* cells calculated from PMA-treated samples can be subtracted from the total number of *B. pertussis* cells to provide the number of dead cells in a sample.

Genomic DNA Isolation

Genomic DNA (gDNA) was isolated using the GenElute Bacteria Genomic DNA Kit (Sigma-Aldrich, Dorset, UK) according to the manufacturer's instructions and eluted with 200 µl of elution buffer. gDNA was purified from THP-1 cells and human challenge model samples using the QIAmp DNA mini and blood extraction kit (QIAgen, Manchester, UK) as per the manufacturer's protocol. gDNA was quantified using a Qubit 1.0 fluorometer (Invitrogen, Loughborough, UK) according to the manufacturer's instructions.

Quantitative PCR

qPCR was performed using a fluorogenic probe (Eurofins, Ebersberg, Germany) targeting insertion sequence IS481. The reaction volume was 20 μl comprising of 2 μl of 1x Taqman Gene Expression Mastermix (Applied Biosystems, Loughborough, UK), 2 μl of 900 nM stocks of each primer, 2 μl of 150 nM stock of probe, 2 μl of nuclease-free water (ThermoFisher Scientific, Invitrogen ™, Loughborough, UK) and 2 μl of template sample. The reactions were run using the StepOne Plus RT PCR System (ThermoFisher Scientific, Loughborough, UK) using the cycling parameters found in Table 1. The sequence of primers and probe were as described previously [30]: forward primer (5′ ATCAAGCACCGCTTTACCC 3′), reverse primer (5′ TTGGGAGTTCTGGTAGGTGTG 3′) and probe (5′ AATGGCAAGGCCGAACGCTTCA 3′) was labelled with FAM and Black Hole Quencher.

Establishing linerarity using standard curves

A 10-fold serial dilution of gDNA in nuclease- free water was assayed for qPCR as described previously. A standard curve was automatically generated using StepOnePlus™ Software v2.3 to establish the linearity of the assay and to allow for the absolute quantification of unknowns.

Calculating copy number from Ct values/ DNA concentration

The genome copy number equivalent to the amount of template in a qPCR reaction was calculated using the formula: copy number = 50 x (amount of template in ng * 6.022×10^{23}) / (length of genome in bp x 1×10^9 * 650). The 50x accounts for the 200 μl volume of extracted gDNA divided by 4 μl of gDNA used in the qPCR reaction. 650 Daltons denotes the average mass of a base pair (bp) and the number of molecules of the template/ gram can be calculated using Avogadro's number, 6.022×10^{23} molecules/mole. The genome of B1917 is 4,102,186 bp [28]. The genome of BP536, UK48, and the mean of all classical *B. pertussis* closed genomes available on RefSeq as of March 2019 was 4.1 mb [22,29,31].

Protocol deposited on protocols.io

The PMA-qPCR method has been deposited on protocols.io and can be accessed here: http://dx.doi.org/10.17504/protocols.io.bc5niy5e.

Preparation of bacterial and THP-1 cell suspensions

To evaluate if eukaryotic cells interfere with the enumeration of live *B. pertussis* cells using qPCR, 10^3 live *B. pertussis* B1917 were combined with THP-1 gDNA equivalent to 10843, 8414, 5385, 3446, 2804, 2316, 1868, 1503, 1251, 1023, 875, 746, 671, 507, 366, or 275, 141, 29 cells. A sample without THP-1 DNA served as a control.

To evaluate the possible sequestration of PMA by eukaryotic DNA, 10^6 heat-killed *B. pertussis* B1917 were combined with either 100,000 heat-killed THP-1 cells, 100,000 live THP-1

Table 1. TaqMan thermocycling conditions for qPCR.

STEP	TEMP	TIME
Step 1 Holding Stage	50˚C	2 minutes
Step 2	95˚C	10 minutes
40 Cycles	95˚C	15 s
	60˚C	60 s

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cells or without THP-1 cells and were then treated with the selected PMA treatment. Non PMA-treated samples were run in parallel.

To determine if eukaryotic cells interfered with the action of PMA on dead bacterial cells, 100,000 live THP-1 cells were combined with different ratios of viable *B. pertussis* cells and heat-killed *B. pertussis* cells (final bacterial concentration was 10^6 cfu/ml) in a clear Eppendorf tube, total volume 200 μl. These samples were then subjected to the selected PMA treatment. A non-PMA treated control was included. gDNA was extracted from each sample and used for qPCR.

Statistical analysis

Unpaired T tests, corrected for multiple comparisons, and two-way ANOVA using the Holm-Sidak method was carried out using Prism 8 for macOS Version 8.2.1 to evaluate statistical significance. One-way ANOVA and Dunnett's multiple comparisons test, with a single pooled variance was also used. A p value of <0.05 was defined as statistically significant and is indicated by asterisks.

Ethics

Samples from volunteers participating in the human challenge model study were obtained in accordance with the provisions of the Declaration of Helsinki (1996) and the International Conference on Harmonization Guidelines for Good Clinical Practice. This study is registered with ClinicalTrials.gov: NCT03751514, was reviewed and approved by the South Central–Oxford A Research Ethics Committee (REC reference: 17/SC/0006, 24 February 2017) and the UK Health Research Authority (IRAS project ID: 219496, 1 March 2017). The protocol has been published previously and details written and oral consent received from human participants [23]. It can be found on www.periscope-project.eu.

Results

qPCR provides a lower limit of detection of 2 *B. pertussis* cells

IS481 is often used as the target for qPCR detection of *B. pertussis* as it is present at ~250 copies per cell in *B. pertussis*, providing great sensitivity. To develop a PMA-qPCR assay, the sensitivity of qPCR for detection of *B. pertussis* was tested over a range of template gDNA concentrations. A linear relationship between Ct value and template concentration was observed over the range of 2 to approximately 2.42×10^6 B1917 cells for qPCR (Fig 1). Ct values greater than 35 were considered to be a negative reaction. Thus, the assay is able to detect *B. pertussis* gDNA equivalent to very few bacterial cells and is linear over a wide range of *B. pertussis* concentrations.

Heat-killing *B. pertussis* at 80˚C for 6 minutes maintained the integrity of cells

The ability of PMA to inhibit PCR-amplification from dead *B. pertussis* was tested using heat-killed *B. pertussis* B1917. It was envisaged that clinical samples may contain dead, but intact, *B. pertussis*. Heat-killing may cause cell lysis which would not mimic intact dead cells. Thus, the integrity of cells following heat killing was assessed using flow cytometry. Incubation of *B. pertussis* suspensions at 80˚C for 6 minutes resulted in 100% killing, but with cells remaining intact and were the conditions used throughout (Fig 2).
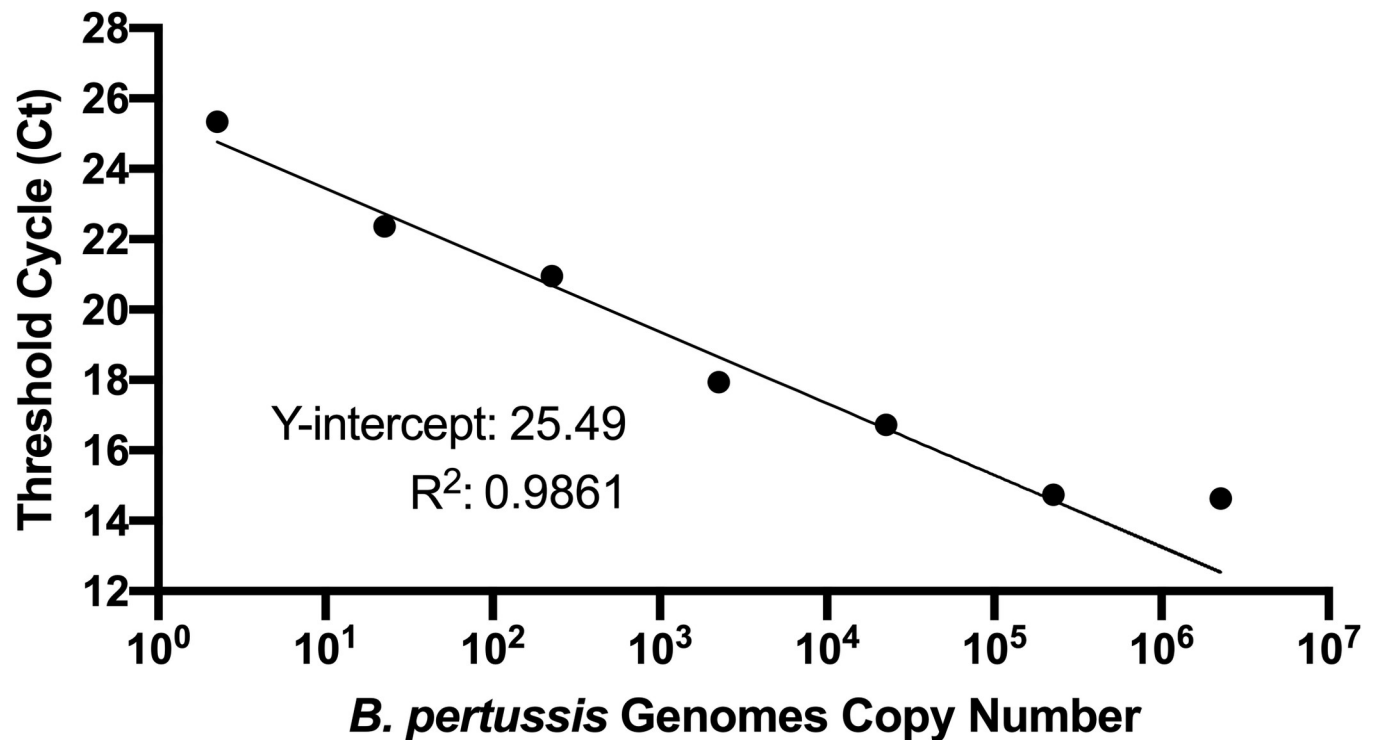
Fig 1. Standard curve of Ct value versus template concentration. Template DNA concentration is expressed as B1917 genome copy number. The linearity was determined to be from 2 to approximately 2.42×10^6 B1917 genomes for qPCR.

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Optimisation of PMA treatment

The effect of PMA concentration on inhibition of PCR amplification from dead *B. pertussis* B1917 was tested (Fig 3). Incubation of heat-killed cells with 50μM of PMA resulted in a

A) Positive Control for Cell Integrity **B) Positive Control for Cell Lysis** **C) Heat-killed at 80°C for 6 minutes**
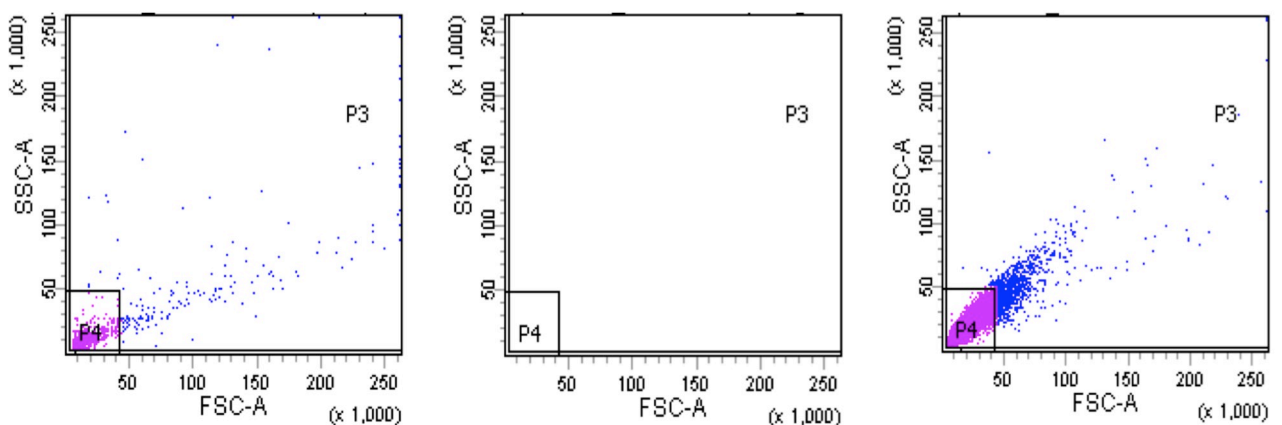

Fig 2. The effect of heat killing on the integrity of *B. pertussis* cells. A) Positive control for cell integrity–a suspension of live *B. pertussis*; B) Positive control for cell lysis–detergent lysed *B. pertussis*; C) Heat-killed *B. pertussis* suspension. The heat-killed *B. pertussis* suspension incubated for 6 minutes at 80°C displayed similar scatter as the live cell suspension. No particles were detected in a suspension of detergent-lysed *B. pertussis*. Therefore, cells remained intact in the heat-killed *B. pertussis* suspension incubated for 6 minutes at 80°C when compared to the positive cell integrity control.

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97.42% reduction in PCR signal compared to that generated from untreated samples. Lower levels of PMA also resulted in very similar levels of inhibition (Fig 3).

The optimal conditions for photo-activation of PMA were determined. Incubation under dark conditions for 10 minutes followed by light activation for between 5 and 30 minutes resulted in greater than 99% reduction in PCR signal from dead cells compared to untreated controls. Five minutes of light activation following 10 minutes of darkness resulted in 99.64% reduction in detection of *B. pertussis* DNA (Fig 4).

From these optimisations, standard conditions of 50μM PMA and incubation in the dark for 10 minutes followed by light activation for 5 minutes were selected as minimal incubation times that achieved high levels of inhibition. Even though 20μM PMA inhibited PCR amplification from dead cells, 50μM PMA was selected as the concentration to use in the assay, as clinical samples will contain cells other than *B. pertussis* that may sequester PMA, requiring an excess for consistent inhibition of PCR signal from dead *B. pertussis*. These conditions were tested in four independent assays. An average of 94.15% reduction in PCR signal was observed compared to untreated controls (Fig 5).

The effect of exogenous cells on detection and PMA-mediated inhibition

Clinical samples are likely to contain cells other than *B. pertussis*, including eukaryotic cells that contain very large amounts of DNA compared to *B. pertussis* cells. Eukaryotic cells may
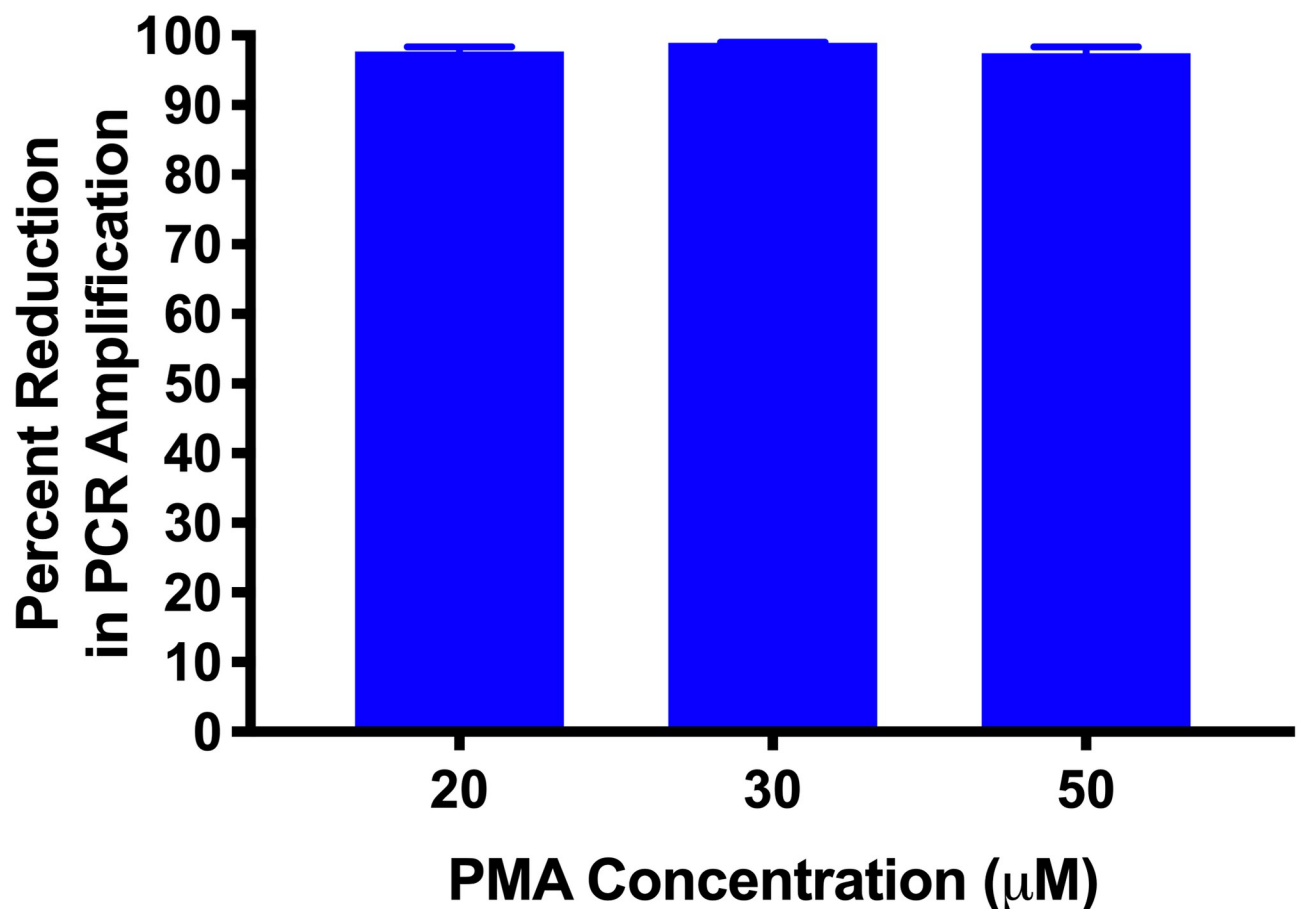

Fig 3. The effect of PMA concentration on the PCR amplification signal from heat-killed cells. Treatment of samples with either 20 μM, 30 μM, or 50 μM of PMA produced a ≥97% reduction in the PCR amplification signal compared to untreated samples. Error bars represent standard deviations from two biological replicates. Data from a representative experiment repeated three times.
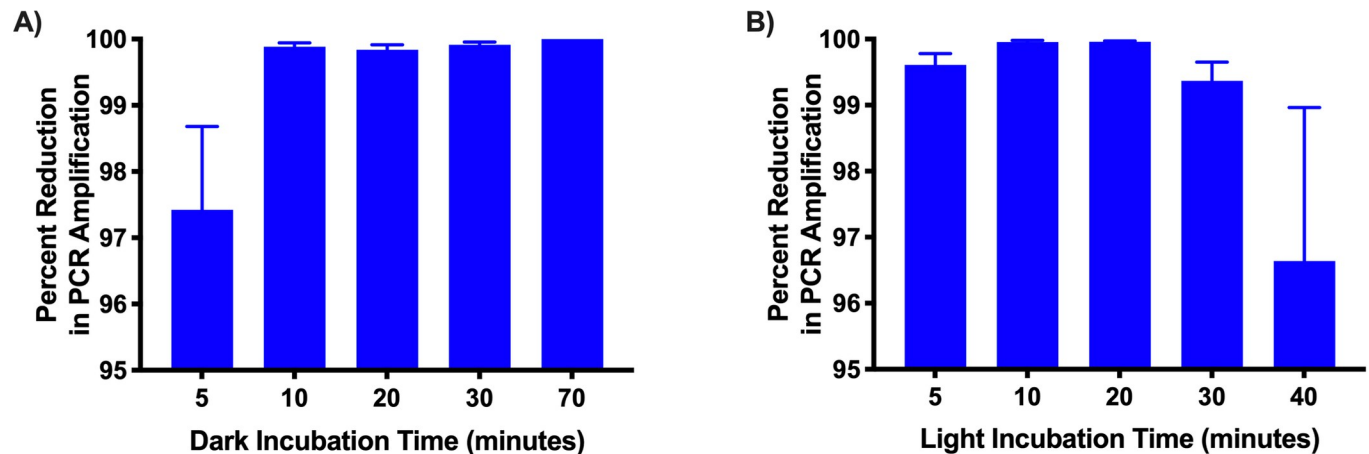
Fig 4. The effect of dark and light exposure times on PMA-inhibition of PCR amplification. A) Dark incubation B) Light Incubation. PMA and untreated heat-killed suspensions were incubated for 10, 20, 30, and 70 minutes in the dark followed by exposure to 5 minutes of light. 10 minutes or longer of incubation in the dark produced a ≥99% reduction in the PCR amplification signal. Optimal light incubation periods were determined by incubating untreated and PMA treated heat-killed suspensions in the dark for 10 minutes followed by light exposure for 5, 10, 20, 30, and 40 minutes. Incubating PMA treated heat-killed samples under light for periods of 5, 10, 20, and 30 minutes produced a 99% or greater reduction in the PCR amplification signal. Five minutes was selected as the standard light incubation period. Error bars represent standard deviation from two biological replicates. The experiment was repeated with the same result.

interfere with the PMA-mediated inhibition of amplification from dead *B. pertussis* preventing distinguishing between live and dead *B. pertussis*. To test this, varying amounts of gDNA from THP-1 cells were combined with a constant amount of *B. pertussis* B1917 gDNA, and Ct values were determined and compared to samples containing *B. pertussis* B1917 only. No effect of THP-1 gDNA on detection of *B. pertussis* was observed up to an equivalent of approximately 5500 THP-1 cells per assay (Fig 6).

It was possible that the presence of other cells would interfere with the PMA-mediated inhibition of PCR signal from dead *B. pertussis*. Thus, the effect of heat-killed or live THP-1 cells on PMA-mediated inhibition of PCR amplification from heat-killed *B. pertussis* was tested. A 99.94% reduction in PCR signal was observed indicating that THP-1 cells did not prevent PMA-mediated inhibition of PCR signal from dead *B. pertussis* (Fig 7).

To test the assay's ability to distinguish between viable and dead *B. pertussis*, in the presence of other cells, a constant number of THP-1 cells were combined with different ratios of heat-killed and viable *B. pertussis* B1917 cells. The reduction in PCR signal was proportional to the amount of heat-killed cells in each suspension (Fig 8) demonstrating that the assay was able to distinguish viable from dead *B. pertussis*, even in the presence of human cells.

Collectively, these studies revealed that the THP-1 cells did not interfere with the PMA-mediated inhibition of PCR signal from dead *B. pertussis* or prevent the accurate enumeration of viable *B. pertussis* cells.

Measuring the viability of *B. pertussis* during in vitro growth

During development of the assay, it was observed that PMA treatment of live *B. pertussis* suspensions used as controls consistently reduced the PCR signal compared to untreated samples. This suggested that *B. pertussis* colonies taken from plate grown cultures contains both live and dead bacteria. To investigate this, and to determine the proportion of live to dead *B. pertussis* in plate grown cultures over time, suspensions of cells were made of *B. pertussis* B1917 grown on plates for either 3, 4, 5 or 8 days. The suspensions were treated with PMA and qPCR
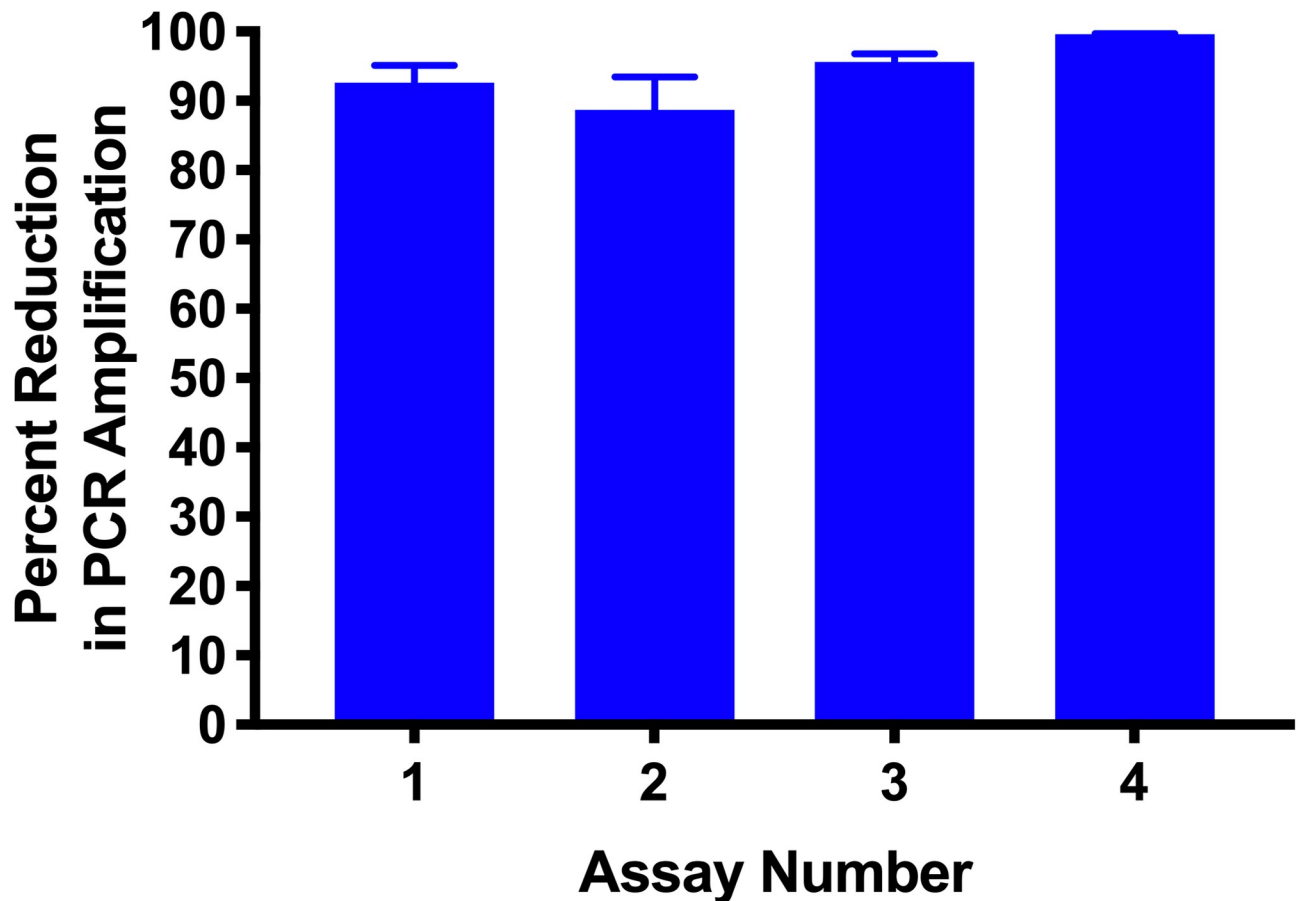
Fig 5. Selected assay conditions gave reproducible inhibition of PCR signal from dead cells. Heat-killed samples were treated with 50 μM of PMA and incubated in the dark for 10 minutes followed by 5 minutes of light activation. A 94.15% reduction in the PCR amplification signal was observed. Error bars represent standard deviations from five biological replicates.

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performed. The percentage of PCR signal observed was compared to untreated controls, Fig 9. *B. pertussis* is relatively slow growing and many protocols for plate growth involve incubation for 72 hours to achieve visible colonies. However, at this point *B. pertussis* viability was only 89%. Interestingly, although colony size continued to increase between days 3 and 5, percentage viability decreased to 24%. Further incubation resulted in further loss in viability. Thus, when using plate grown *B. pertussis* in assays, suspensions will be a mixture of live and dead bacteria, and that enumeration of *B. pertussis* by plating serial dilutions of a suspension and counting the resulting CFU's will not be a measure of the total number of cells in the suspension.

Use of the assay to enumerate live and dead *B. pertussis* from human challenge model samples

The assay was developed in order to provide a method for monitoring the colonisation status of participants in a novel human challenge model of *B. pertussis* colonisation. During development of this model, a group of participants were inoculated with 10^5 CFU of *B. pertussis* B1917 and daily samples were taken over a 14-day period to monitor colonisation, including nasosorption fluids, pernasal swabs, throat swabs, and nasopharyngeal washes [21].
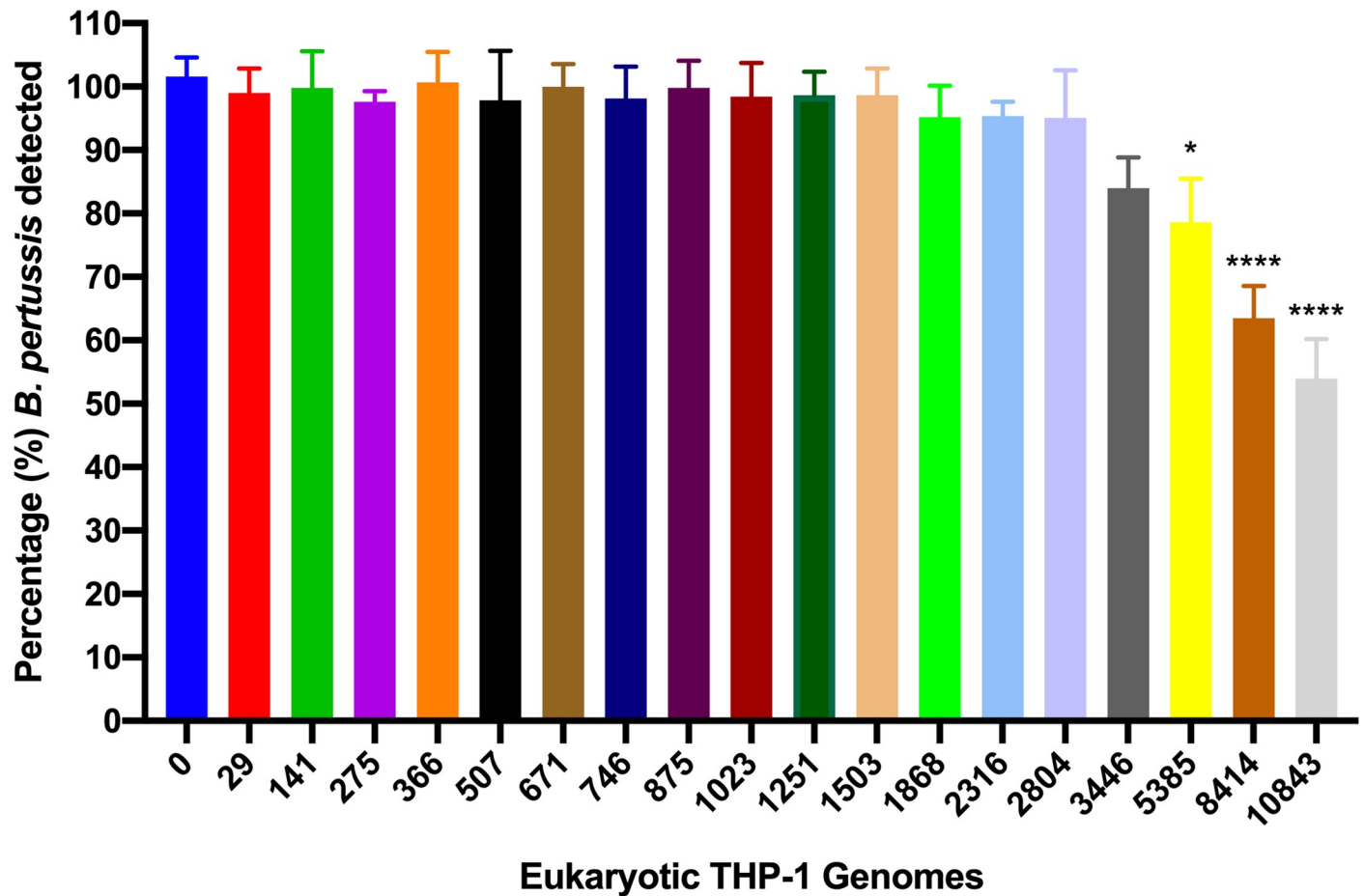
Fig 6. Effect of eukaryotic gDNA on the detection of *B. pertussis*. Varying amounts of gDNA from THP-1 cells were combined with gDNA equivalent to 10^3 *B. pertussis* cells. No interference in detection of *B. pertussis* was observed up to the equivalent of 3446 THP-1 cells, after which the sensitivity of detection was reduced when compared to viable *B. pertussis* detected in the presence of 0 THP-1 cells. *: $p < 0.05$, determined by one-way ANOVA and Dunnett's multiple comparisons test, with a single pooled variance. Error bars represent standard deviations from three biological replicates.

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Here, samples from Day 9 post-challenge were tested by PMA-qPCR, and by culture, Fig 10. Samples were split and one portion was treated with PMA. *B. pertussis* were enumerated using qPCR from PMA-treated and untreated samples. Using culture, 3 out of 5 participants were determined to be colonised [21]. By PMA-qPCR, 4 out of 5 volunteers were deemed to carry viable *B. pertussis*, with detection from nasal washes, pernasal swabs, nasosorption and throat samples. Nasal washes from Day 11 samples also had detectable viable *B. pertussis* in 2 of the 5 volunteers using PMA-qPCR and in a third volunteer using culture. (Fig 10E). Pernasal samples from Day 11 revealed detectable *B. pertussis* in a single volunteer using culture, that was not detected using PMA-qPCR (Fig 10F). PMA-qPCR revealed samples contained both viable and dead *B. pertussis*, in approximately equal numbers. Interestingly, on Day 16 of sampling, two days after volunteers started azithromycin treatment to eradicate the infection, all but one volunteer was negative for detectable *B. pertussis* cells. In this volunteer, the PMA-qPCR assay was able to detect low levels of viable and dead *B. pertussis*, with a higher proportion of dead genomes detected compared to viable genomes, however these low levels of *B. pertussis* were undetectable by culture (Fig 10G). Nasosorptions from this cohort of volunteers were all culture-negative.
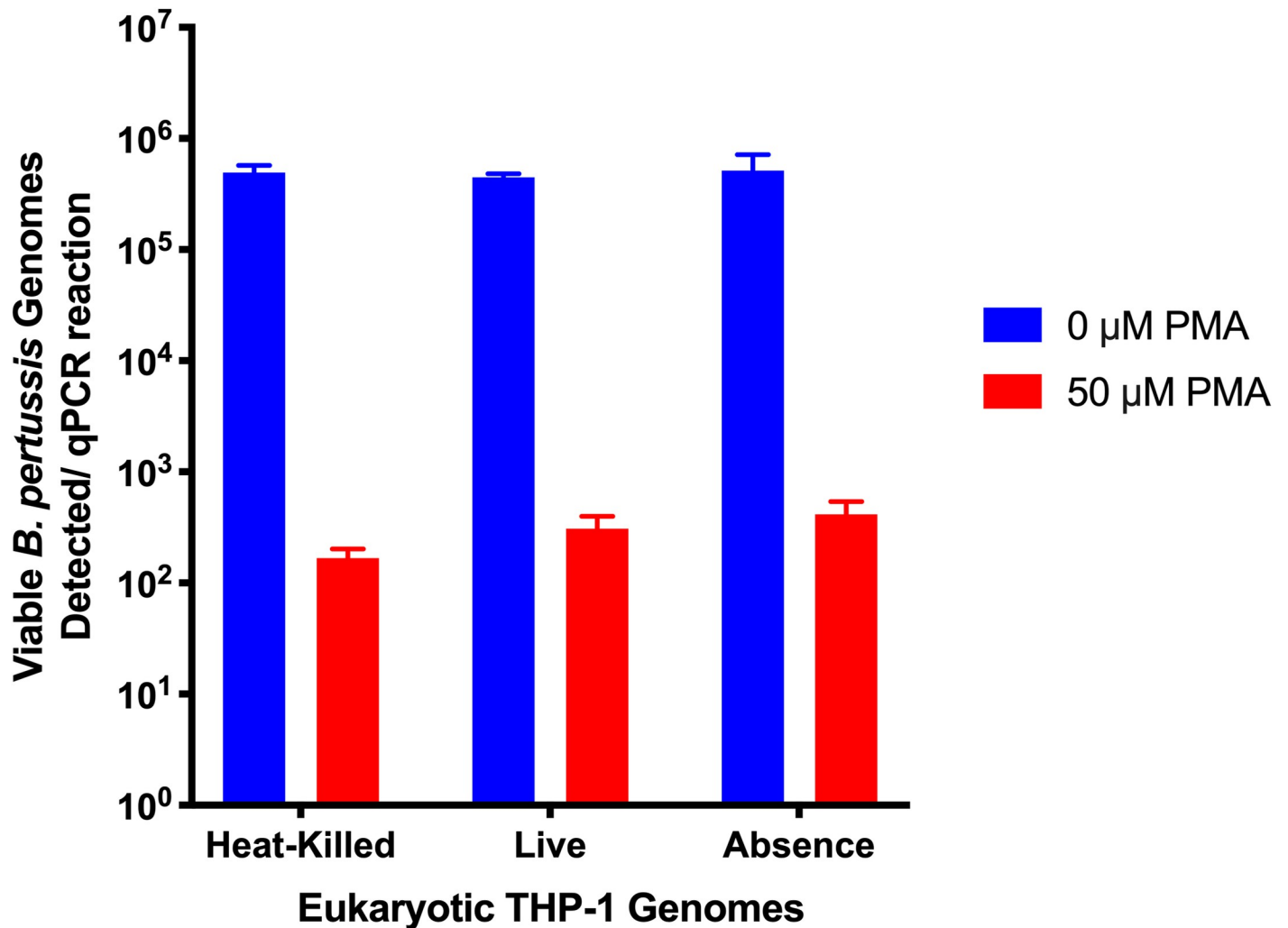
Fig 7. Eukaryotic THP-1 gDNA did not interfere with enumeration of *B. pertussis*. gDNA from 10^6 heat-killed *B. pertussis* were combined with either 10^5 heat-killed THP-1 cells, 10^5 live THP-1 cells or a no THP-1 cell control and treated with PMA. Non PMA-treated samples were run in parallel. The presence of live or dead THP-1 cells did not interfere with the action of PMA on dead *B. pertussis* cells. Error bars represent standard deviations from three biological replicates.

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Confirming utility of PMA-qPCR assay by enumerating five additional strains of *B. pertussis*

To confirm the utility of this assay with other strains outside of the human challenge model, qPCR was performed on gDNA extracted from PMA and non-PMA treated suspensions from the following strains: BP536, UK48, UK71, B204 (B1878), B184 (B2973). B1917 was also enumerated as a control (Fig 11). These suspensions contained *B. pertussis* cells taken from three-day old plate cultures resuspended in PBS to an OD_{600} = 1.0. The suspensions were plated onto agar to confirm the enumeration obtained by the PMA-qPCR assay. The recovery of viable *B. pertussis* cells from both PMA-qPCR and culture were comparable for all strains, confirming the utility of this assay as a reliable method for enumeration, however, there was a significant increase in the recovery of viable cells from B1917 and BP536 using PMA-qPCR compared to culture. DNA copy numbers were calculated using the mean genome size of 4.1 mb.
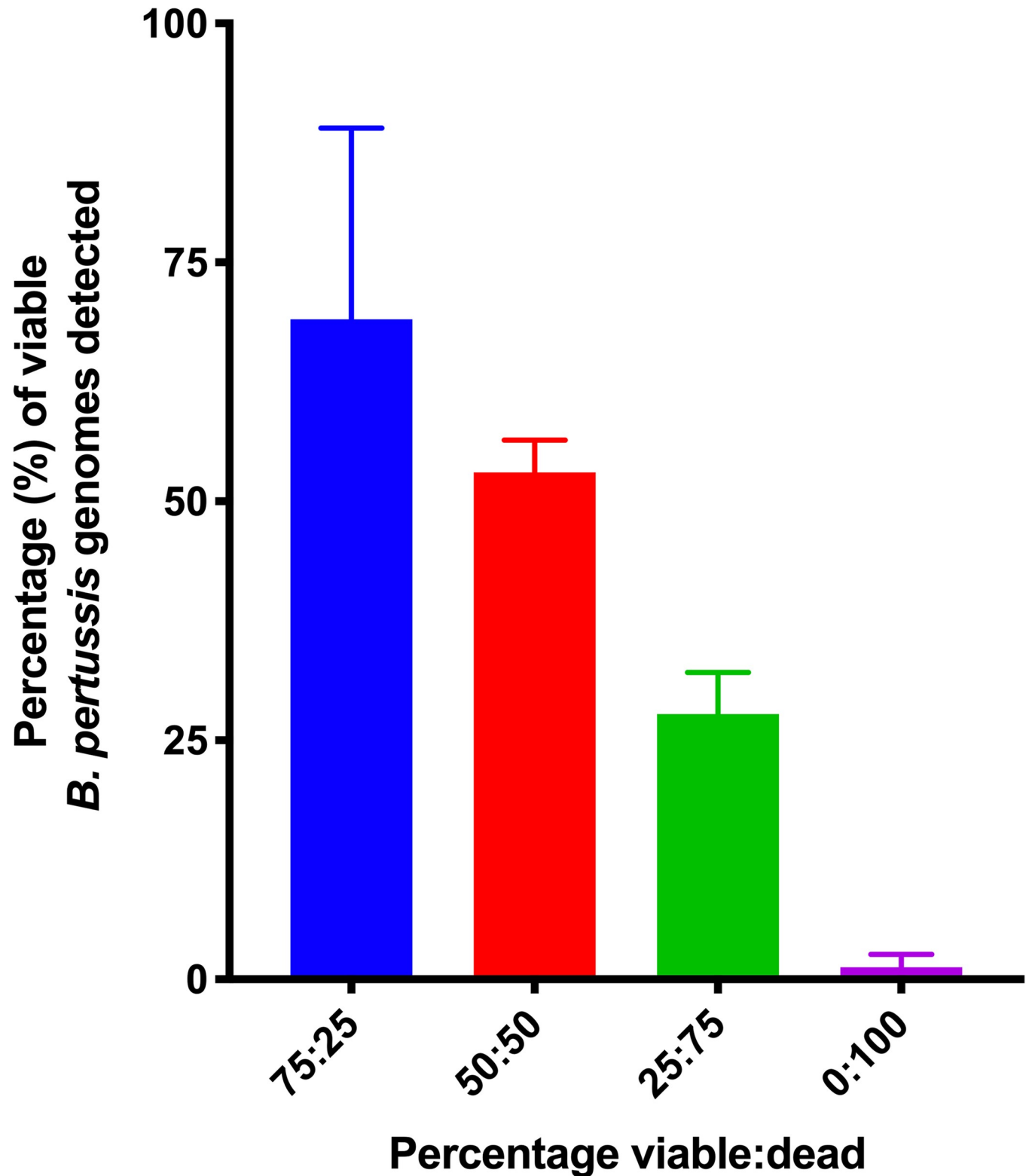
Fig 8. Viable *B. pertussis* cells enumerated from PMA treated samples in the presence of eukaryotic cells. 100,000 THP-1 cells were combined with suspensions of different ratios of heat-killed and viable *B. pertussis* cells. The assay accurately distinguished viable from dead *B. pertussis* in each suspension. Error bars represent standard deviations from three biological replicates.
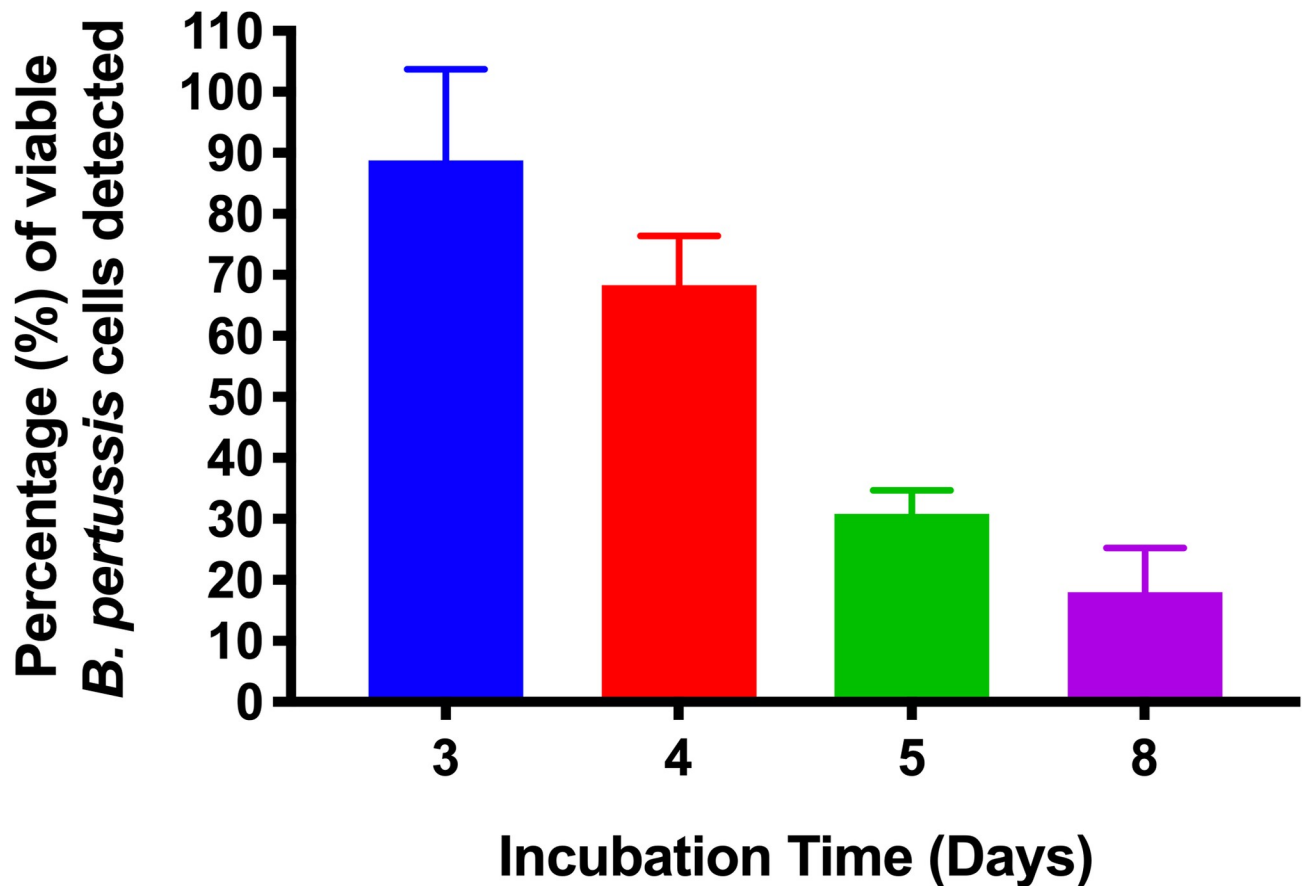
https://doi.org/10.1371/journal.pone.0232334.g008

Fig 9. The viability of *B. pertussis* decreases during growth on agar plates. The viability of *B. pertussis* growing on agar plates was measured over time. Viability decreased as the incubation time increased with only 24% of cells being viable after 5 days of incubation. Error bars represent standard deviations from three biological replicates.

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Discussion

Ordinarily, the detection and quantification of viable *B. pertussis* is achieved through culture on laboratory agar. However, the relatively slow growth rate of *B. pertussis* means that the growth of countable colonies can take between 72–120 hours. The development of a human challenge model for *B. pertussis* as part of the PERISCOPE project requires that enumeration of viable *B. pertussis* be achieved in a much shorter time than this, in order to be able follow colonisation closely.

In addition, simple enumeration of viable bacteria within a sample doesn't provide the complete picture. In many scenarios, such as measuring bacterial load in an infection model, it is of great interest to know the total bacterial number as understanding the dynamics of bacterial growth that involves both cell division and cell death is very important. Thus, while traditional qPCR provides a faster detection method for *B. pertussis* than culture, the modification of a qPCR assay with the introduction of PMA treatment of samples reported here enables both fast detection of *B. pertussis* and the ability to distinguish viable from dead cells.

Here, we demonstrate that PMA inhibits PCR-mediated amplification from dead *B. pertussis* and that inhibition of signal from dead cells occurs even in the presence of high numbers of eukaryotic cells. This may be important for the detection of *B. pertussis* from complex samples that contain a mix of cell types as seen in the human challenge model. Samples obtained from
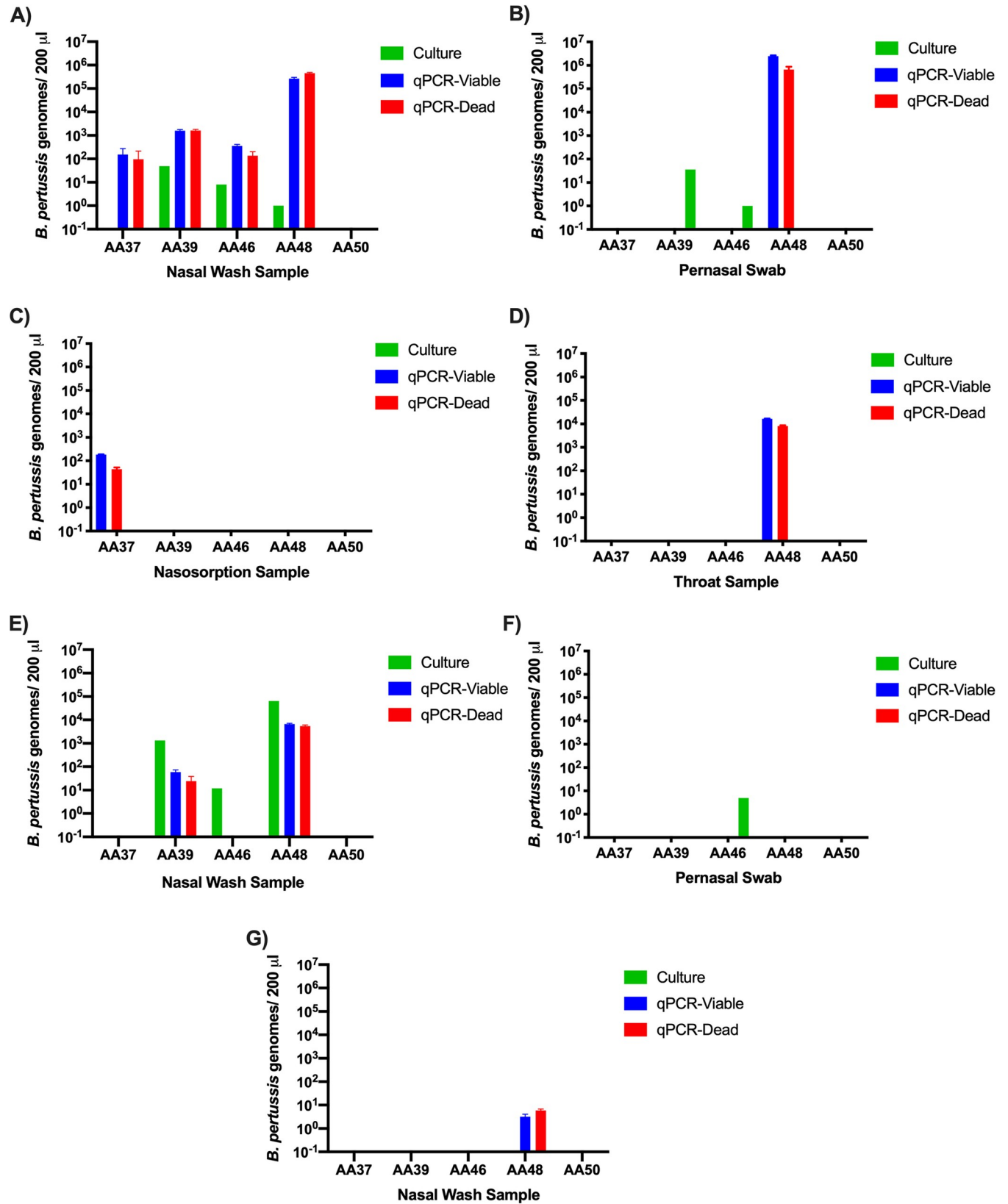
Fig 10. PMA-qPCR detected viable *B. pertussis* from human challenge model samples within hours compared to culture. Viable and dead *B. pertussis* were enumerated in samples from 5 volunteers in the human challenge model, collected Day 9 (A-D), Day 11 (E-F) and Day 16 (G) after inoculation, from the sample type indicated. Two hundred µl of samples were processed. Day 16 samples are taken two days after volunteers started azithromycin treatment to clear infection. Values below the lower limit of detection were considered undetectable and given a value of 0.

volunteers that were identified as positive for *B. pertussis* by culture, were also detected in our initial test of the PMA-qPCR assay. The same volunteers were identified as being negative for *B. pertussis* by both qPCR and culture, with the exception of a single sample that had low levels of *B. pertussis* identified only by qPCR. This result demonstrates the high sensitivity of this assay to detect very low levels of viable and dead *B. pertussis*. Further optimisation studies to determine the exact point in which there is loss in sensitivity when amplifying fewer than 10^3 *B. pertussis* cells in the presence of THP1 cells would further support the results obtained from the human challenge model. Interestingly, PMA-qPCR detected approximately equal numbers of viable and dead *B. pertussis*, demonstrating its use to enumerate total bacteria rather than only viable cells. The full results of the human challenge model are published elsewhere [21]. Here we demonstrate that the PMA-qPCR assay allowed for a determination of colonisation status within hours of obtaining the samples compared to days when using culture.

To confirm that the assay can be used with strains other than B1917, we tested five additional *B. pertussis* strains. Approximately 250 copies of IS481 were found in all isolates of *B. pertussis*, however, the exact number of copies differs amongst strains within a narrow range
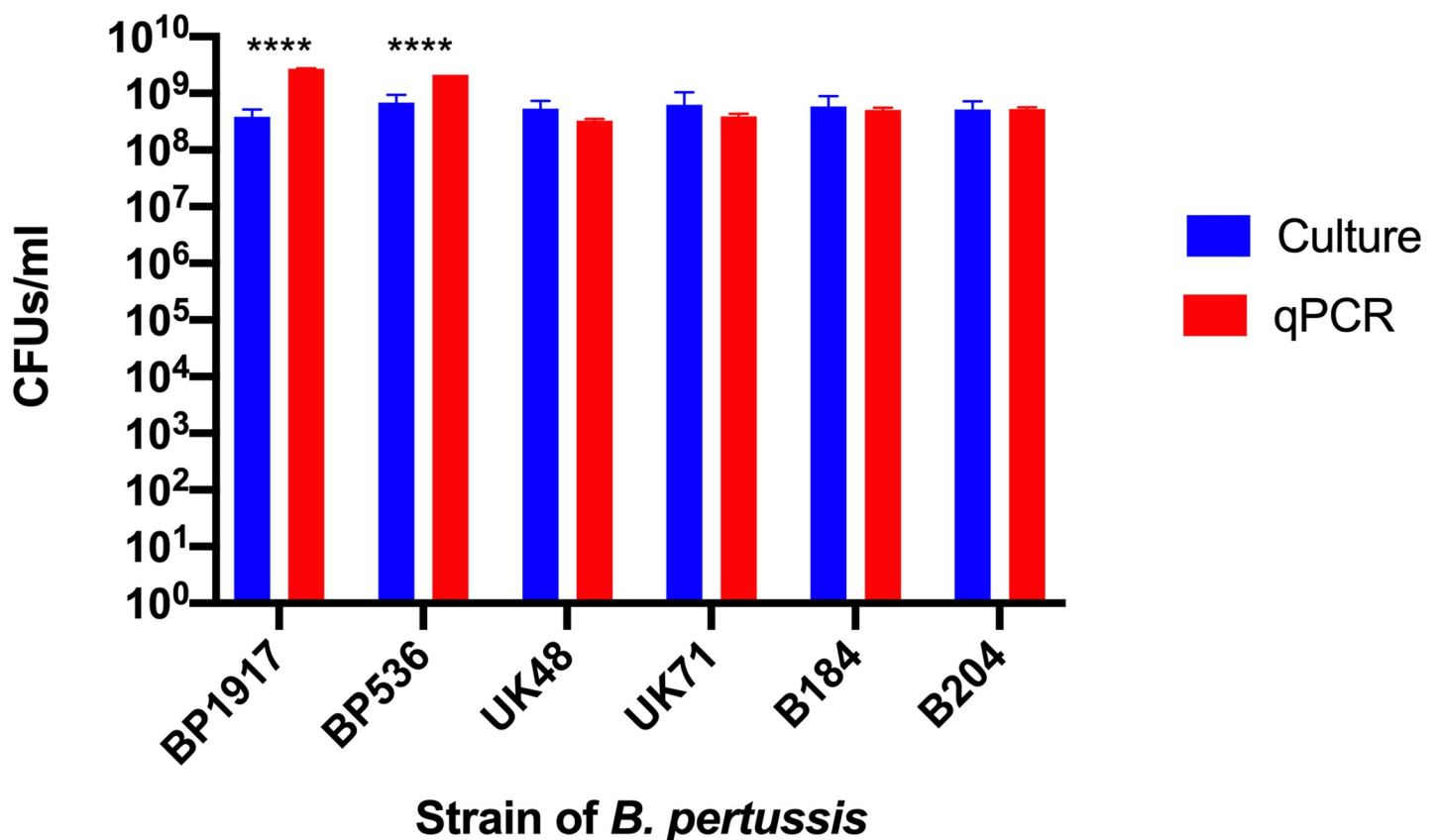

Fig 11. Enumeration of six strains of *B. pertussis* with both culture and PMA-qPCR produced comparable values. The exceptions were B1917 and BP536 for which a higher number of viable *B. pertussis* cells were detected using the PMA- qPCR assay. Error bars represent standard deviations from three biological replicates.

[30]. The number of copies range from 236–272 among the closed genome sequences available for *B. pertussis*. Thus, this will create some error when performing absolute quantification of strains for which the copy number is not known, but this error is not large (<10%).

The use of IS481 as a target means that there is the chance of cross-reactivity of IS481 with *B. holmseii* and *B. bronchiseptica*, although *B. holmseii* is rarely recovered from nasopharyngeal samples [32]. However, here, this assay was specifically designed to support the human challenge model, therefore using a single qPCR target of IS481 to detect known amounts of *B. pertussis* B1917 that has been administered to volunteers was appropriate. To extend the use of this assay and to increase specificity, species-specific targets, which are commonly used in many diagnostic labs, should be considered. [30]. These changes will reduce false positives and result in a more sensitive and specific assay for the accurate diagnosis of *B. pertussis*, as well as help rule out coinfections or pertussis symptoms caused by other *Bordetella spp*.

The utility of the PMA-qPCR assay has been shown for the human challenge model, but has wider uses. For example, in diagnostic laboratories, where ascertaining if *B. pertussis* is viable or dead will facilitate whether to pursue culture as a means to obtaining a live culture for characterisation. It is also of use in a range of research and industrial settings enabling investigation of the dynamics of *B. pertussis* growth by determining both cell division and cell death.

Supporting information

S1 Table. Standard curve of Ct value versus template concentration.
(XLSX)

S2 Table. The effect of PMA concentration on the PCR amplification signal from heat-killed cells.
(XLSX)

S3 Table. The effect of dark and light exposure times on PMA-inhibition of PCR amplification. A) Dark Incubation B) Light Incubation.
(XLSX)

S4 Table. Selected assay conditions gave reproducible inhibition of PCR signal from dead cells.
(XLSX)

S5 Table. Effect of eukaryotic gDNA on the detection of *B. pertussis*.
(XLSX)

S6 Table. Eukaryotic THP-1 gDNA did not interfere with enumeration of *B. pertussis*.
(XLSX)

S7 Table. Viable *B. pertussis* cells enumerated from PMA treated samples in the presence of eukaryotic cells.
(XLSX)

S8 Table. The viability of *B. pertussis* decreases during growth on agar plates.
(XLSX)

S9 Table. PMA-qPCR detected viable *B. pertussis* from human challenge model samples within hours compared to culture. A) Day 9- Nasal washes B) Day 9- Pernasal Swab C) Day 9- Nasosorption D) Day 9- Throat E) Day 11- Nasal washes F) Day 11- Pernasal swabs G) Day 16- Nasal washes.
(XLSX)

S10 Table. Enumeration of six strains of *B. pertussis* with both culture and PMA-qPCR produced comparable values.
(XLSX)

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References

1. Mattoo S, Cherry JD. Clinical Manifestations of Respiratory Infections Due to Bordetella pertussis and Other Bordetella Subspecies Molecular Pathogenesis, Epidemiology, and Clinical Manifestations of Respiratory Infections Due to Bordetella pertussis and Other Bordetella Su. Clin Microbiol Rev. 2005; 18(2):326–82. https://doi.org/10.1128/CMR.18.2.326-382.2005 PMID: 15831828

2. Kilgore PE, Salim AM, Zervos MJ, Schmitt H. Pertussis: Microbiology, Disease, Treatment, and Prevention. 2016; 29(3):449–86.

3. Wagner B, Melzer H, Freymüller G, Stumvoll S, Rendi-Wagner P, Paulke-Korinek M, et al. Genetic variation of bordetella pertussis in Austria. PLoS One. 2015; 10(7):1–13.

4. HPA. Confirmed pertussis cases (England and Wales). Heal Prot Rep. 2012; 6(43).

5. Spokes PJ, Quinn HE, McAnulty JM. Review of the 2008–2009 pertussis epidemic in NSW: notifications and hospitalisations. N S W Public Health Bull. 2010; 21(8):167.

6. De Melker HE, Schellekens JFP, Neppelenbroek SE, Mooi FR, Rümke HC, Conyn-Van Spaendonck MAE. Reemergence of pertussis in the highly vaccinated population of the Netherlands: Observations on surveillance data. Emerg Infect Dis. 2000; 6(4):348–57. https://doi.org/10.3201/eid0604.000404 PMID: 10905967

7. Kanojia G, Raeven RHM, van der Maas L, Bindels THE, van Riet E, Metz B, et al. Development of a thermostable spray dried outer membrane vesicle pertussis vaccine for pulmonary immunization. J Control Release [Internet]. 2018; 286(July):167–78. Available from: https://doi.org/10.1016/j.jconrel.2018.07.035

8. Van der Maas NAT, Mooi FR, de Greeff SC, Berbers GAM, Spaendonck MAEC, de Melker HE. Pertussis in the Netherlands, is the current vaccination strategy sufficient to reduce disease burden in young infants? Vaccine [Internet]. 2013; 31(41):4541–7. Available from: https://doi.org/10.1016/j.vaccine.2013.07.060 PMID: 23933365

9. Winter K, Harriman K, Zipprich J, Schechter R, Talarico J, Watt J, et al. California Pertussis epidemic, 2010. J Pediatr [Internet]. 2012; 161(6):1091–6. Available from: https://doi.org/10.1016/j.jpeds.2012.05.041 PMID: 22819634

10. Acosta AM, DeBolt C, Tasslimi A, Lewis M, Stewart LK, Misegades LK, et al. Tdap Vaccine Effectiveness in Adolescents During the 2012 Washington State Pertussis Epidemic. Pediatrics. 2015; 135 (6):981–9. https://doi.org/10.1542/peds.2014-3358 PMID: 25941309

11. Warfel JM, Merkel TJ. Bordetella pertussis infection induces a mucosal IL-17 response and long-lived Th17 and Th1 immune memory cells in nonhuman primates. Mucosal Immunol [Internet]. 2012; 6 (4):787–96. Available from: https://doi.org/10.1038/mi.2012.117 PMID: 23187316

12. Ross PJ, Sutton CE, Higgins S, Allen AC, Walsh K, Misiak A, et al. Relative Contribution of Th1 and Th17 Cells in Adaptive Immunity to Bordetella pertussis: Towards the Rational Design of an Improved Acellular Pertussis Vaccine. PLoS Pathog. 2013; 9(4).

13. Althouse BM, Scarpino S V. Asymptomatic transmission and the resurgence of Bordetella pertussis. BMC Med [Internet]. 2015; 13(1):1–12. Available from: http://dx.doi.org/10.1186/s12916-015-0382-8

14. Zeddeman A, van Gent M, Heuvelman CJ, van der Heide HG, Bart MJ, Advani A, et al. Investigations into the emergence of pertactin-deficient Bordetella pertussis isolates in six European countries, 1996 to 2012. Euro Surveill Bull Eur sur les Mal Transm = Eur Commun Dis Bull. 2014; 19(33):1–11.

15. Bodilis H, Guiso N. Virulence of pertactin-negative Bordetella pertussis isolates from infants, France. Emerg Infect Dis. 2013; 19(3):471–4. https://doi.org/10.3201/1903.121475 PMID: 23621904

16. Otsuka N, Han HJ, Toyoizumi-Ajisaka H, Nakamura Y, Arakawa Y, Shibayama K, et al. Prevalence and genetic characterization of pertactin-deficient bordetella pertussis in Japan. PLoS One. 2012; 7(2):1–7.

17. Lam C, Octavia S, Ricafort L, Sintchenko V, Gilbert GL, Wood N, et al. Rapid increase in pertactin-deficient Bordetella pertussis isolates, Australia. Emerg Infect Dis. 2014; 20(4):626–33. https://doi.org/10.3201/eid2004.131478 PMID: 24655754

18. Pawloski LC, Queenan AM, Cassiday PK, Lynch AS, Harrison MJ, Shang W, et al. Prevalence and molecular characterization of pertactin-deficient Bordetella pertussis in the United States. Clin Vaccine Immunol. 2014; 21(2):119–25. https://doi.org/10.1128/CVI.00717-13 PMID: 24256623

19. Safarchi A, Octavia S, Luu LDW, Tay CY, Sintchenko V, Wood N, et al. Pertactin negative Bordetella pertussis demonstrates higher fitness under vaccine selection pressure in a mixed infection model. Vaccine [Internet]. 2015; 33(46):6277–81. Available from: https://doi.org/10.1016/j.vaccine.2015.09.064 PMID: 26432908

20. Diavatopoulos DA, Mills KHG, Kester KE, Kampmann B, Silerova M, Heininger U, et al. PERISCOPE: road towards effective control of pertussis. Lancet Infect Dis. 2019; 19(5):e179–86. https://doi.org/10.1016/S1473-3099(18)30646-7 PMID: 30503084

21. De Graaf H, Ibrahim M, Hill AR, Gbesemete D, Vaughan AT, Gorringe A, et al. Controlled human infection with Bordetella pertussis induces asymptomatic, immunising colonisation. Clin Infect Dis [Internet]. 2019 Sep 28; Available from: https://doi.org/10.1093/cid/ciz840

22. Ring N, Abrahams JS, Bagby S, Preston A, MacArthur I. How Genomics Is Changing What We Know About the Evolution and Genome of Bordetella pertussis. Adv Exp Med Biol. 2019; 1183:1–17. https://doi.org/10.1007/5584_2019_401 PMID: 31321755

23. Wu B, Liang W, Kan B. Enumeration of viable non-culturable Vibrio cholerae using propidium monoazide combined with quantitative PCR. J Microbiol Methods [Internet]. 2015; 115:147–52. Available from: https://doi.org/10.1016/j.mimet.2015.05.016 PMID: 26001818

24. Nocker A, Cheung CY, Camper AK. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods. 2006; 67(2):310–20. https://doi.org/10.1016/j.mimet.2006.04.015 PMID: 16753236

25. Taylor MJ, Bentham RH, Ross KE. Limitations of Using Propidium Monoazide with qPCR to Discriminate between Live and Dead Legionella in Biofilm Samples. Microbiol insights [Internet]. 2014; 7(7):15–24. Available from: http://www.la-press.com/limitations-of-using-propidium-monoazide-with-qpcr-to-discriminate-bet-article-a4365

26. Ruike W, Higashimori A, Yaguchi J, Li Y -y. Use of real-time PCR with propidium monoazide for enumeration of viable Escherichia coli in anaerobic digestion. Water Sci Technol [Internet]. 2016; 74 (5):1243–54. Available from: https://doi.org/10.2166/wst.2016.327 PMID: 27642844

27. Nkuipou-Kenfack E, Engel H, Fakih S, Nocker A. Improving efficiency of viability-PCR for selective detection of live cells. J Microbiol Methods [Internet]. 2013; 93(1):20–4. Available from: https://doi.org/10.1016/j.mimet.2013.01.018 PMID: 23389080

28. Bart MJ, Zeddeman A, van der Heide HG, Heuvelman K, van Gent M, Mooi FR. Complete Genome Sequences of Bordetella pertussis Isolates B1917 and B1920, Representing Two Predominant Global

Lineages. Genome Announc [Internet]. 2014; 2(6):2–3. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25540342

29.   Parkhill J, Sebaihia M, Preston A, Murphy LD, Thomson N, Harris DE, et al. Comparative analysis of the genome sequences of bordetella pertussis, bordetella parapertussis and bordetella bronchiseptica. Nat Genet. 2003; 35(1):32–40. https://doi.org/10.1038/ng1227 PMID: 12910271

30.   Probert WS, Ely J, Schrader K, Atwell J, Nossoff A, Kwan S. Identification and evaluation of new target sequences for specific detection of Bordetella pertussis by real-time PCR. J Clin Microbiol. 2008; 46 (10):3228–31. https://doi.org/10.1128/JCM.00386-08 PMID: 18753352

31.   Ring N, Abrahams JS, Jain M, Olsen H, Preston A, Bagby S. Resolving the complex Bordetella pertussis genome using barcoded nanopore sequencing. Microb genomics. 2018; 4(11).

32.   Martini H, Detemmerman L, Soetens O, Yusuf E, Piérard D. Improving specificity of Bordetella pertussis detection using a four target real-time PCR. PLoS One. 2017; 12(4):1–11.