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Development of a rapid recombinase polymerase amplification assay for the detection of *Streptococcus pneumoniae* in whole blood

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**Abstract**

**Background:** *Streptococcus pneumoniae* is an important cause of microbial disease in humans. The introduction of multivalent vaccines has coincided with a dramatic decrease in the number of pneumococcal-related deaths. In spite of this, at a global level, pneumococcal infection remains an important cause of death among children under 5 years of age and in adults 65 years of age or older. In order to properly manage patients and control the spread of infection, a rapid and highly sensitive diagnostic method is needed for routine implementation, especially in resource-limited regions where pneumococcal disease is most prevalent.

**Methods:** Using the gene encoding leader peptidase A as a molecular diagnostics target, a real-time RPA assay was designed and optimised for the detection of *S. pneumoniae* in whole blood. The performance of the assay was compared to real-time PCR in terms of its analytical limit of detection and specificity. The inhibitory effect of human genomic DNA on amplification was investigated. The potential clinical utility of the assay was investigated using a small number of clinical samples.

**Results:** The RPA assay has a limit of detection equivalent to PCR (4.0 and 5.1 genome equivalents per reaction, respectively) and was capable of detecting the equivalent of <1 colony forming unit of *S. pneumoniae* when spiked into human whole blood. The RPA assay was 100 % inclusive (38/38 laboratory reference strains and 19/19 invasive clinical isolates) and 100 % exclusive; differentiating strains of *S. pneumoniae* species from other viridans group streptococci, including *S. pseudopneumoniae*. When applied to the analysis of a small number (n = 11) of clinical samples (blood culture positive for *S. pneumoniae*), the RPA assay was demonstrated to be both rapid and sensitive.

**Conclusions:** The RPA assay developed in this work is shown to be as sensitive and as specific as PCR. In terms of reaction kinetics, the RPA assay is shown to exceed those of the PCR, with the RPA running to completion in 20 minutes and capable generating a positive signal in as little as 6 minutes. This work represents a potentially suitable assay for application in point-of-care settings.

**Keywords:** *Streptococcus pneumoniae*, Recombinase Polymerase Amplification, Leader peptidase A, Molecular diagnostics
Background

Streptococcus pneumoniae is a major cause of human disease. It is estimated that more than 1.6 million people, including more than 800,000 children under the age of 5, die every year from pneumococcal infections [1]. In addition to its role as a leading cause of community-acquired pneumonia, S. pneumoniae is a causative agent of a variety of other infections, including otitis media, blood stream infection, spontaneous peritonitis and meningitis [2]. Pneumococcal meningitis is a form of pneumococcal disease associated with significant mortality and morbidity in children. In developed countries, pneumococcal meningitis has a mortality rate of 17–30 % [3] and an associated risk of sequelae of 32 % [4]. In developing countries, the mortality and morbidity rates are much higher [5]. S. pneumoniae belongs to the mitis subgroup of alpha-haemolytic (viridans) streptococci (VGS). Mitis group streptococci other than S. pneumoniae are also associated with bacteriaemia which may be clinically non-significant but may also be associated with invasive disease, particularly in predisposed patients such as the immunocompromised and those with cardiac valve damage [6]. The mitis group organisms, S. mitis, S. oralis and S. pseudopneumoniae, possess >99 % 16S rRNA sequence homology with S. pneumoniae [7]. Furthermore, horizontal gene transfer between streptococci can give rise to atypical streptococci that are difficult to classify using phenotypic methods [8]. Given the close genotypic and phenotypic relationship between mitis group streptococci, the differentiation of pneumococcus from other VGS is challenging [9].

Culture based methods are the gold standard for diagnosing invasive pneumococcal disease (IPD). However culture based methods typically require at least several hours to a day or more incubation before an organism is detected. Furthermore, these methods often demonstrate limited diagnostic sensitivity, particularly post antibiotic treatment or in cases with low sample volume, as is often the case with children [10, 11]. Molecular amplification methods such as PCR and Loop-mediated isothermal amplification (LAMP) have been used to diagnose IPD [12, 13]. These methods are reported as sensitive, are not reliant on having viable organisms and have the potential to deliver more rapid results.

Recombinase polymerase amplification (RPA) is an isothermal in vitro nucleic acid amplification technology that uses a combination of recombinase protein, oligonucleotide primers and a strand displacing polymerase to amplify DNA sequences [14]. RPA is sensitive, rapid, specific and robust (less sensitive to inhibitors than PCR). RPA has been applied to the detection of a wide variety of bacterial, viral and eukaryotic molecular targets [15–22]. The use of a fluorescent probe enables monitoring of reactions in real-time and multiplexing capabilities. Isothermal amplification strategies are of great interest in molecular diagnostics, as they negate the need for thermocycling, as is the case for PCR. The isothermal nature of these techniques makes them suitable for incorporation into devices capable of being deployed at the point-of-care.

Molecular targets that have been used to identify S. pneumoniae include a variety of genes, including the Spn9802 fragment [23], the RecA gene [24] the 16S rRNA gene [25], and virulence factor genes, such as autolysin (lytA) [26] and pneumolysin (ply) [27, 28]. Whilst these targets have proven useful for the detection of S. pneumoniae, their ability to unequivocally identify S. pneumoniae remains problematic. For example, false positives can occur with the lytA marker [29] and both ply [30] and Spn9802 [23] are associated with false negative results. Leader peptidase A (LepA), also known as Elongation Factor 4 (EF4), is one of the most conserved bacterial proteins and is found in virtually all known genomes [31]. It functions as a ribosome dependant GTPase, with the ability to back-translocate post-translocational ribosomes and increase the active fraction of newly translated proteins [32–34]. Its utility as a diagnostic marker has been previously demonstrated in a multiplex assay for the detection of the Mycobacterium tuberculosis complex (MTC) [35, 36].

In this study, we have developed and performed a preliminary evaluation of a real-time RPA assay based on the LepA gene for the detection of S. pneumoniae. We also developed a LepA based S. pneumoniae real-time PCR assay to compare in terms of specificity and sensitivity.

Methods

Bacterial strains and growth conditions

S. pneumoniae bacterial reference strains (n = 8), non-S. pneumoniae reference strains (n = 54) and invasive S. pneumoniae clinical isolates (n = 19) were evaluated in this study (Table 1). All strains were cultured overnight in brain heart infusion (BHI) medium (Oxoid, UK) at 37 °C. For enumeration, 100 μL of serially diluted bacterial suspensions were spread plated on Columbia blood agar and incubated for 24 hrs.

DNA extraction

Following overnight culture, bacterial genomic DNA was extracted and purified using the DNeasy Blood and Tissue kit (Qiagen, Germany) according to the manufacturers’ instructions. Following purification, DNA was quantified by fluorescence (Qubit DNA BR Assay, Life technologies, UK). Genome equivalents (GE) were calculated based on an assumed genome size of 2.1 Mb [37]. For whole blood spiking experiments, from an overnight
Table 1 Bacterial strains and reactivity in the RPA and PCR assays

| Organism          | Strain ID | RPA  | PCR  |
|-------------------|-----------|------|------|
| S. pneumoniae DM  | 20566     | +    | +    |
| S. pneumoniae DM  | 11865     | +    | +    |
| S. pneumoniae DM  | 11866     | +    | +    |
| S. pneumoniae DM  | 14377     | +    | +    |
| S. pneumoniae DM  | 24048     | +    | +    |
| S. pneumoniae DM  | 11868     | +    | +    |
| S. pneumoniae DM  | 25971     | +    | +    |
| S. pneumoniae DM  | 14378     | +    | +    |
| S. pneumo BCCM   | 15081     | -    | -    |
| S. pneumo BCCM   | 15082     | -    | -    |
| S. pneumo BCCM   | 15083     | -    | -    |
| S. pneumo BCCM   | 15084     | -    | -    |
| S. pneumo BCCM   | 15085     | -    | -    |
| S. pneumo BCCM   | 15086     | -    | -    |
| S. pneumo BCCM   | 15087     | -    | -    |
| S. pneumo BCCM   | 15094     | -    | -    |
| S. pneumo BCCM   | 15095     | -    | -    |
| S. anginosus BCCM| 20563     | -    | -    |
| S. australis BCCM| 15627     | -    | -    |
| S. bovis BCCM    | 20480     | -    | -    |
| S. canis BCCM    | 20715     | -    | -    |
| S. constellatus B| 20575     | -    | -    |
| S. cristatus DSM  | 8249      | -    | -    |
| S. downei DSM     | 5365      | -    | -    |
| S. dysgalactiae B| 6176      | -    | -    |
| S. equi DSM       | 20561     | -    | -    |
| S. equis DSM      | 20554     | -    | -    |
| S. gordonii DSM   | 6777      | -    | -    |
| S. infantis DSM   | 12492     | -    | -    |
| S. intermedius DSM| 20573    | -    | -    |
| S. mitis DSM      | 12643     | -    | -    |
| S. mutans DSM     | 20523     | -    | -    |
| S. oralis DSM     | 20066     | -    | -    |
| S. parasanguinis B| 6778      | -    | -    |
| S. perosis DSM    | 12493     | -    | -    |
| S. porcinus DSM   | 20725     | -    | -    |
| S. pseudopneumonia DSM 18670 | - | - |
| S. pyogenes DSM   | 2072      | -    | -    |

Table 1 Bacterial strains and reactivity in the RPA and PCR assays (Continued)

| Organism          | Strain ID |
|-------------------|-----------|
| S. pyogenes DSM   | 20565     |
| S. salivarius DSM | 20560     |
| S. salivarius DSM | 20617     |
| S. sanguinis DSM  | 20567     |
| S. sinensis DSM   | 14990     |
| S. suis DSM       | 9682      |
| S. uberis DSM     | 20569     |
| S. vestibularis DSM| 5636     |
| H. influenzae DSM | 4690      |
| H. influenzae DSM | 11121     |
| H. parainfluenzae DSM| 8978   |
| H. haemolyticus CCG| 15312   |
| H. somnus CCG     | 12839     |
| N. meningitidis DSM| 10036    |
| K. pneumoniae DSM | 30184     |
| P. aeruginosa DSM | 50071     |
| E. coli DSM       | 30083     |
| E. faecalis DSM   | 20317     |
| C. albicans CBS   | 2700      |
| B. fragilis DSM   | 2151      |
| M. catarrhalis DSM| 11994     |
| S. aureus DSM     | 346       |

DSM Leibniz-Institut, DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, BCCM Belgian Coordinated Collections of Microorganisms, CCG Culture Collection, University of Göteborg, Sweden

culture, the culture medium was 10-fold serially diluted in BHI. 2 μL of the serially diluted bacterial suspensions, were spiked into 98 μL of fresh human whole blood. Subsequently, total genomic DNA was extracted and purified using the DNeasy Blood and Tissue kit (Qiagen, Germany) according to the manufacturers’ instructions, with the exception that DNA was eluted in 20 μL H2O (rather than 200 μL buffer AE).

Primer and probe design

LepA sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank), and the Functional Gene Pipeline and Repository (FunGene; http://fungene.cme.msu.edu) website. Sequences were aligned using the multiple sequence alignment tool ClustalW [38]. Following alignment, S. pneumoniae specific PCR and RPA primers and probes were manually designed (Table 2). The sequences were finally screened for homology using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm their specificity. All oligonucleotide primers and probes were purchased from Integrated DNA Technologies (Leuven, Belgium), with the exception of the RPA probe,
which was purchased from Biosearch Technologies (Petaluma, California, USA).

**Real-time recombinase polymerase amplification**

RPA reactions were performed using the TwistAmp Exo® kit (TwistDx, Cambridge, UK) and contained: 1 x rehydration buffer, 4 μL forward and reverse primer (6 μM each), 4 μL probe (6 μM) and 1 μL genomic DNA as the amplification template. To initiate the reaction, 14 mM magnesium acetate (2.5 μL at a concentration of 280 mM) was added. Reactions were performed in a total volume of 50 μL at 40 °C in a portable real-time fluorometer (Twista®, TwistDx) for 20 minutes with a mixing and centrifugation step after the first 4 minutes. External negative and positive amplification controls were included in each run. The positive amplification control consisted of 100 GE of the *S. pneumoniae* type strain (DSM 20566) diluted in molecular grade water. The no-template control (NTC) consisted of molecular grade water.

**Real-time PCR**

All real-time PCR assays were performed using a LightCycler® 480 real-time PCR instrument (Roche Diagnostics, UK). PCR reactions were performed in 200 μL 12-well optical strips (BIOplastics BV, The Netherlands) in a total volume of 20 μL. Each reaction consisted of 1 x LightCycler Probes Master mix (Roche Diagnostics, UK), 10pmoles each primer and 5pmoles of probe and 1 μL genomic DNA as the amplification template. Negative and positive amplification controls were included in each run. The positive amplification control consisted of 100 GE of the *S. pneumoniae* type strain (DSM 20566) diluted in molecular grade water. The no template control (NTC) consisted of molecular grade water. The cycling parameters consisted of 1 cycle of 95 °C for 5mins followed by 45 cycles of 95 °C for 10s, 60 °C for 15 s and 72 °C for 1 s.

**Sensitivity analysis/determination**

The analytical limit of detection (95 % confidence level) of the PCR and RPA assays were determined. *S. pneumoniae* (DSM 20566) genomic DNA was diluted in molecular grade water to 8, 7, 6, 5, 4, 3, 2, or 1 GE per microliter. PCR and RPA reactions were then performed using 1 μL of the diluted DNA as the amplification template. Reactions were performed in replicates of twelve and the subsequent data was analysed statistically (Probit, Minitab, Version 16).

**Evaluation of inhibition of RPA by human DNA**

In order to determine the limit of background human DNA tolerated by the RPA and PCR reactions, varying quantities of human genomic DNA (100, 200 and 400 ng) were added to reactions containing 50, 20 or 4 GE of *S. pneumoniae* (DSM 20566) DNA.

**Table 2 Nucleic Acid sequences of primers and probes**

| Probe/primer | DNA sequence (5'-3') | Nucleotide position | Amplicon size (bp) |
|--------------|----------------------|---------------------|-------------------|
| RPA Forward  | ACAGCTCCGCTCTGTATTACAAAGTTAATTTA*C   | 1123–1157          | 190               |
| RPA Reverse  | AGTCCCAACCTTACGTGCTAGCTCCATTAC*T      | 1278–1312          |                   |
| RPA Probe    | CTTGACATAAGGTCTCTAATGCTGCAATCT[T(FAM)][A(dSpacer)] [T(BHQ-1)][TGGGCTCTGGAAC]*T | 1193–1242          |                   |
| PCR Forward  | CTCGTAAAGCGTAAACTCTTGT                   | 1706–1727          | 107               |
| PCR Reverse  | CATACCTAGGCCGTGAGGA                    | 1793–1813          |                   |
| PCR Probe    | FAM-ACGCATGAAATCCATCAGGAGT-TAMRA        | 1749–1773          |                   |

*aThe nucleotide position refers to the LepA gene from *S. pneumoniae* strain SPNA45 (Genbank accession number NC_018594.1)

*b*indicates a phosphorothioate bond

**Specificity analysis/determination**

The specificity of both the RPA and PCR assays were evaluated. Genomic DNA (5 × 10⁴ GE) from a panel of *Streptococcus* and non-*Streptococcus* organisms (see Table 1) was tested in both RPA and PCR amplification assays.

**Blood spiking experiments**

Human whole blood was collected from a healthy volunteer in EDTA vacutainers (Becton Dickinson and Company, Ireland). Two microliters of 10-fold serially diluted bacterial suspensions (over five orders of magnitude) were spiked into 98 μL of fresh (within 1 hr of sampling) human whole blood. Aliquots (100 μL) of the serially diluted bacterial suspensions were plated on blood agar for enumeration. The spiked blood samples were then processed immediately to extract DNA. From the eluted DNA (20 μL), 1 μL was then subjected to RPA or PCR amplification.
Clinical sample analysis using RPA and PCR
Whole blood was collected in EDTA blood tubes from patients attending Galway University Hospital, suspected of a bloodstream infection for routine full blood count and from healthy volunteers. Generally, within 24 hours of collection of patient blood, residual material was collected, anonymised and then stored at −80 °C. Blood collected from the healthy volunteers was also stored at −80 °C. Subsequently, the samples were thawed on ice and total genomic DNA was extracted from 100 μL of sample and eluted in 20 μL molecular grade water as described previously. For analysis, 1 μL or 5 μL of the eluted genomic DNA was subjected to amplification by RPA and PCR.

Ethical approval
Ethical approval for this study was granted by the Ethics Committee of the Galway University Hospital, Galway, Ireland. Participants were selected on the basis of having blood cultures submitted to the Department of Medical Microbiology for detection of bloodstream infection. Following full blood count, patients for whom residual EDTA blood samples were available were recruited into the study based on the results of the blood culture (confirmed positive for the presence of S. pneumoniae). For controls, blood was collected from healthy individuals, following informed consent.

Results
Analytical specificity
The analytical specificities of both the RPA and real-time PCR assays were determined and are shown in Table 1. Both assays detected the 3 S. pneumoniae references strains tested. Both assays also detected all invasive clinical isolates (n = 19). Of the 39 other Streptococcus and non-Streptococcus species that were tested, none were detected by either of the assays, indicating the analytical specificity of the assays (Table 1, Fig. 1).

Analytical limit of detection
Genomic DNA was serially diluted (100–1 GE per reaction) in water and subjected to RPA. Initial experiments suggested that the RPA was capable of detecting 1 GE per reaction (Fig. 2). Subsequently, the analytical limit of detection of both the RPA and real-time PCR assays were measured by testing 12 replicates of serially diluted genomic DNA from the S. pneumoniae type strain (DSM 20566). The analytical limit of detection (95 % confidence level) of the RPA and PCR assays was established to be 4.1 and 5.1 GE per reaction, respectively. Table 3 outlines the number of reactions producing a positive result for each template quantity.

Assessment of inhibition and detection in whole blood
The degree to which the RPA reaction was inhibited by the presence of human DNA was concentration dependant, varying from no significant inhibition (100 ng; data

![Fig. 1 RPA assay specificity. The graph shows the fluorescence intensity of an assay containing 100 genome equivalents (GE) of S. pneumoniae (type strain; DSM20566), 5 x 10⁶ GE of 6 streptococcus species and a no template control (NTC) ](image-url)
not shown) to substantial inhibition in the presence of 400 ng human DNA (the highest tested). Whilst inhibited, when spiked with 200 ng or 400 ng of human DNA, 50, 20 and 4 S. pneumoniae GE were detected, all producing signals above that of the negative control reaction (Fig. 3). When the PCR reaction was spiked with human DNA (100, 200 or 400 ng/reaction), there was no discernible shift in threshold-cycle between spiked and non-spiked reactions, indicating that the PCR was not inhibited by the presence of human DNA (data not shown).

The ability of the assays to detect S. pneumoniae spiked into whole blood from a healthy volunteer was also determined. Plate counts indicated that 0.0185, 0.185, 1.85, 18.5, 185 CFU had been spiked into 98 μL of blood. Upon extraction, total DNA was eluted into 20 μL, of which 1 μL was subjected to RPA and PCR, meaning that each reaction contained template genomic DNA from 0.000925, 0.00925, 0.0925, 0.925 or 9.25 CFU equivalents. Both the RPA and PCR assays detected 0.925 and 9.25 CFU equivalents per reaction (Fig. 4). The lower quantities (0.000925, 0.00925, 0.0925 CFU) were not detected.

**Detection of S. pneumoniae in clinical samples**

Eleven blood samples confirmed by culture to be positive for S. pneumoniae were subjected to analysis by RPA and PCR. Both the RPA and PCR assays were positive for the same 8 of the 11 samples tested (Table 4). For the remaining 3 S. pneumoniae positive samples, 5 μL of the purified DNA was subsequently tested in the RPA and PCR assays, resulting in positive signals. Figure 5 shows representative RPA amplification curves from the resultant DNA purified from 4 confirmed positive clinical blood samples. RPA and PCR analysis of DNA extracted from the blood of four healthy (culture negative) volunteers, produced negative results.

**Discussion**

Some 93 polysaccharide capsular types or serotypes of S. pneumoniae have been identified [39]. The introduction of a 7-, 13-, and more recently, a 23-valent conjugate vaccine has been associated with a dramatic reduction in...
the number of deaths, particularly amongst children and in adults (via a herd effect) due to pneumococcal infection [40, 41]. However, pneumococcal disease remains a serious disease globally, particularly in children less than two years old, the elderly (>65 years) and in immunocompromised individuals. Thus, a robust diagnostic assay capable of the rapid, sensitive and specific detection of *S. pneumoniae* in near-patient settings could play a significant role in the reduction of pneumococcal disease related morbidity and mortality, particularly in developing countries.

Isothermal *in vitro* nucleic acid amplification strategies are of great interest in molecular diagnostics, as they negate the need for thermocycling, as is the case for PCR. In PCR, thermocycling is required to facilitate the separation of double-stranded DNA to enable amplification. The major isothermal amplification techniques currently utilised include: Nucleic Acid Sequence based Amplification (NASBA) [42], Transcription Mediated Amplification (TMA) [43], Loop-Mediated Isothermal Amplification (LAMP) [44], Helicase Dependent Amplification (HDA) [45], Rolling Circle Amplification (RCA) [46] and Strand Displacement Amplification (SDA) [47]. Of these, NASBA, TMA, RCA and SDA cannot be considered truly isothermal as they require an initial heating step to denature the target nucleic acid prior to amplification. RPA, HDA and LAMP can be considered truly isothermal, as there is no requirement for a denaturation step to initiate amplification. The operating temperature of LAMP is typically 60–65 °C, at which temperature the nucleic acid target is in dynamic equilibrium, enabling the binding of primers to their target sequence and subsequent amplification. Whilst LAMP is inexpensive to perform, primer design is complex and multiplex amplification is at present difficult. Both HDA and RPA use proteins to facilitate the binding of primers to their respective template and both technologies enable

![Figure 3](image-url)
multiplex amplification. The major advantage of RPA over other competing isothermal amplification technologies is its speed, being capable of amplifying single-digit copy numbers of nucleic acids to detectable levels in 5–10 minutes.

In this study, we have developed real-time RPA and real-time PCR assays for the detection of *S. pneumoniae* using primers and probes targeting the *LepA* gene. We examined the ability of both assays to detect *S. pneumoniae* spiked into human whole blood. Finally, we applied both assays to the detection of *S. pneumoniae* directly in the blood of 11 patients confirmed positive for infection by blood culture.

Both RPA and PCR assays were highly specific, with all laboratory strains (*n* = 8) and clinical isolates of *S. pneumoniae* (*n* = 19) positively identified. Both assays were negative for all non-*S. pneumoniae* organisms (*n* = 39) assayed. These data support the results of our *in silico* analysis, demonstrating that the *LepA* gene has sufficient sequence heterogeneity to enable the specific detection of *S. pneumoniae*. Probit regression analysis was used to calculate (with 95% confidence) the analytical LOD of both assays. The RPA assay had a marginally better LOD than the PCR assay (4.0 and 5.1 GE respectively). Assuming Poisson distribution, both assays demonstrated limits of detection approaching 3 molecules, the most sensitive LOD theoretically possible [48]. It should also be noted that the RPA reactions were performed in volumes of 50 μL, 250% greater than the 20 μL PCR reactions, suggesting that by reducing the reaction volume and thereby increasing the effective concentration of the initial template DNA, the assay sensitivity could be further improved.

RPA is a rapid isothermal *in vitro* nucleic acid amplification technology. When 10 *S. pneumoniae* GE were

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**Table 4** Analysis of clinical samples. RPA and PCR assay reactivity

| Sample IDa | RPA / PCRb |
|------------|------------|
|            | 1 μL | 5 μL |
| BS 12/2013/1 | +   |    |
| BS 12/2013/2 | +   |    |
| KH 12/2013/1 | -   | +  |
| KH 12/2013/2 | +   |    |
| DK 10/2011/2 | +   |    |
| MG 03/2012 | +   |    |
| EM 03/2012 | -   | +  |
| MF 03/2012 | -   | +  |
| AF 02/2012/1 | +   |    |
| AF 02/2012/2 | +   |    |
| MR 04/2013/2 | +   |    |
| HC1 | -   |    |
| HC2 | -   |    |
| HC3 | -   |    |
| HC4 | -   |    |

*a* Samples HC1, HC2, HC3 and HC4 were obtained from healthy individuals

*b* 1 or 5 μL of purified DNA was required to produce a positive signal in the RPA or PCR

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**Fig. 4** Blood spiking. The graph shows the fluorescence intensity obtained from the RPA of 1 μL purified DNA (9.25, 0.925 and 0.0925 CFU equivalents) extracted from whole blood spiked with varying CFU of *S. pneumoniae* (type strain; DSM20566). Also shown is the fluorescence intensity obtained from the amplification of 100 GE (positive control, PC) of *S. pneumoniae* (type strain; DSM20566) and a no template control reaction (NTC).
used as the template for amplification, our RPA assay produced a positive signal in as little as 5 mins. This is in contrast to 35 cycles (approximately 20mins), which was required to produce a positive signal in our PCR assay for the equivalent template quantity.

Prior to performing our spiking experiments we investigated what affect the presence of background human DNA had on the RPA assay performance. We found that in the presence of 400 ng background DNA (the highest quantity tested), although considerably inhibited, the RPA assay was capable of detecting 50, 20 and 4 GE, all giving signals above the no template control reaction. In the presence of 200 ng of background human DNA, the RPA assay was also inhibited, but to a much lesser degree than in the presence of 400 ng DNA. According to the DNA extraction kit manufacturer (Qiagen), 3–6 μg is the typical yield of genomic DNA obtained from 100 μL of mammalian blood. The DNA we used for the inhibition studies was extracted using the DNeasy blood and tissue kit and yields were in this range (data not shown). Assuming the average yield is 4.5 μg DNA /100 μL blood, and considering that we eluted our extracted DNA into 20 μL, of which 1 μL is subjected to RPA, the quantity of background human DNA in the RPA reaction is approximately 225 ng. We also tested the effect of background human DNA on the PCR assay and found that the reaction was not inhibited, even in the presence of 400 ng of background DNA.

When spiked into whole blood, both PCR and RPA assays were capable of detecting 0.925 CFU/reaction, which equals 18.5 CFU equivalents/100 μL blood. Reactions in the blood spiking experiment were performed in duplicate and subsequently repeated to verify this result. Peter et al. (2009) developed a real-time PCR assay targeting the autolysin gene capable of detecting the equivalent of 125 CFU in 1 mL whole blood [49]. It should be noted, that CFU is an estimate of the number of viable organisms present. CFU as a metric does not account for the presence of dead, non-viable cells or for organisms that tend to form clumps or grow as pairs or chains and may well substantially underestimate the number of living cells in a sample [50].

To further evaluate our RPA assay, we tested a small number of *S. pneumoniae* culture positive (n = 11) and culture negative (obtained from consenting volunteers) blood samples (n = 4). All culture positive samples resulted in positive signals when analysed by RPA and PCR amplifications while the four culture negative samples were RPA and PCR negative. Eight of the 11 culture positive samples produced positive signals for RPA and PCR when tested with 1 μL of purified DNA. The remaining three that tested negative using 1 μL of purified DNA, were then tested using 5 μL of purified DNA in both assays, with both assays producing positive signals. This result hints at a reduced bacterial DNA load or the presence of RPA and PCR reaction inhibitors in the 3 of the 11 culture positive samples in question. This may have been the result of lower bacterial cell load in the samples, DNA degradation due to extended sample processing time during sample acquisition.
Conclusions
We have developed a sensitive and specific real-time RPA and a real-time PCR assay for the detection of S. pneumoniae in clinical blood samples. We have shown this assay to be as specific and as sensitive as PCR when applied to the detection of S. pneumoniae using the LepA gene as a diagnostics target. This assay may find utility as a rapid point-of-care diagnostic test for the detection of S. pneumoniae. Future work will focus on the development of an internal amplification control for the assay and increasing the number of clinical samples to fully validate the RPA assay.

Competing interests
We declare the following competing interests; TwistDx supplied RPA reagents for the study. MSF and OP are employees of TwistDx. TB and TJS are named inventors on a patent application pertaining to the use of LepA as a diagnostics target. OP is a named inventor of Recombinase Polymerase Amplification technology.

Authors’ contributions
OH, EC, MSF, OP, TB and TJS designed the study. OH performed the experimental work. EC drafted the manuscript. TBW and MC had roles collection and analysis of clinical isolates and samples. All authors contributed to the critical review and revision of the manuscript. All authors have seen and approved the final version.

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