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Article

Multiplex Recombinase Polymerase Amplification Assay for Simultaneous Detection of *Treponema pallidum* and *Haemophilus ducreyi* in yaws-like lesions

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Abstract: Yaws is a skin debilitating disease caused by *Treponema pallidum* subspecies *pertenue* with most cases reported in children. World Health Organization (WHO) aims at total eradication of this disease through mass treatment of suspected cases followed by an intensive follow-up program. However, effective diagnosis is pivotal in the successful implementation of this control program. Recombinase polymerase amplification (RPA), an isothermal nucleic acid amplification technique offers a wider range of differentiation of pathogens including those isolated from chronic skin ulcers with similar characteristics such as *Haemophilus ducreyi* (*H. ducreyi*). We have developed a duplex RPA assay for the simultaneous detection of *Treponema pallidum* (*T. pallidum*) and *H. ducreyi* (TPHD-RPA). TPHD-RPA assay demonstrated no cross-reaction with other pathogens and enable detection of *T. pallidum* and *H. ducreyi* within 15 minutes at 42 °C. The duplex RPA assay was validated with 49 clinical samples from individuals confirmed to have yaws by serological tests. Compared with commercial multiplex real-time PCR, the TPHD-RPA assay demonstrated 94-95% sensitivity for *T. pallidum* and *H. ducreyi* confirmed samples, respectively and 100% specificity. This simple novel TPHD-RPA assay enables the rapid detection of both *T. pallidum* and *H. ducreyi* in yaws-like lesions. This test could support the yaws eradication programs by ensuring effective diagnosis as well as enable monitoring of eradication efforts success or failure and planning of follow-up interventions at the community level.

Keywords: Recombinase polymerase amplification; *Treponema pallidum*; *Haemophilus ducreyi*; Molecular diagnostics; Point-of-care test

1. Introduction

Yaws, most common of the three endemic non-venereal treponemal infections is caused by *Treponema pallidum* sp. *pertenue* [1, 2]. Yaws initially present as lesions of the skin (papilloma), which ulcerates and progresses to cause destructive lesions of bone and cartilage leading to chronic disfigurement and disability when left untreated. The transmission is via direct (non-sexual)

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contact with fluid exudate from the lesion of an infected person [3]. The disease mostly affects children living in poor communities of West Africa, Latin America, Asia, and the Pacific where access to healthcare is limited [3]. Estimates in 2012 show that over 89 million people are affected with Yaws in 15 countries in Africa, Southeast Asia, and the Pacific region [4].

The WHO aimed to achieve total eradication of yaws by 2020 through a comprehensive large-scale treatment strategy, called the Morges strategy [5]. This strategy consists of an initial mass drug therapy with oral intake of azithromycin in endemic communities followed by an active surveillance every 6 months to actively detect and treat remaining cases and their contacts. Pilot implementation of this eradication strategy in endemic countries such as Ghana, Congo, Papua New Guinea, Vanuatu, and the Solomon Islands have shown promising results, with the need for scale-up (Asiedu, Fitzpatrick, & Jannin, 2014), [6]. However, the success of the Morges eradication strategy is hinged on the selection of the most appropriate diagnostic assay at each stage of the eradication effort to ensure a complete halt in the transmission of yaws [7]. The occurrence of *Haemophilus ducreyi*, as a common cause of chronic skin ulcers similar to yaws in endemic areas also compounds the challenges of diagnosing yaws [8, 9].

Traditional serological tests such as Treponema Pallidum Particle Agglutination assay (TPPA) and Rapid Plasma Reagin test (RPR) have remained the common diagnostic tool for yaws, over the years [1]. These tools present issues of low sensitivity and specificity hence are not appropriate for use during the follow-up stage of the eradication strategy [10]. The Dual Path Platform (DPP) Syphilis Screen-and-Confirm assay (Chembio Diagnostic Systems, Inc., NY, USA), which enables the simultaneous detection of antibodies to treponemal and non-treponemal antigens are accurate for the confirmation of clinically suspected cases in yaws-endemic communities. This point-of-care (POC) test has increased sensitivity in high seropositive individuals and low in individuals with low titre serology [10]. Low titre serology is evident after mass drug administration, hence DPP may also not be effective in follow up case detection. Additionally, issues of co-infections cannot be overlooked as reactive syphilis serology caused by latent yaws has been observed in ulcers with *H. ducreyi* [8]. Also, the WHO recommends the use of molecular diagnostic tests as part of the case definition in yaws eradication efforts (7). These highlights the importance of a more sensitive and specific diagnostic test in confirming yaws suspected cases even after mass drug administration.

Nucleic acid amplification technique especially polymerase chain reaction (PCR) can help address all issues with yaws diagnosis due to its high clinical sensitivity and specificity to *T. pallidum* and *H. ducreyi*. This test could play a central role in the yaws eradication strategy by ensuring effective diagnosis and surveillance after MDA in yaws-endemic communities [7, 8, 11]. However, this technique can only be applied in a reference laboratory and not at the point of care in endemic communities. As such, the need for diagnostic techniques that do not involve costly equipment and can be used in remote areas have been indicated [12]. The use of isothermal amplification techniques such as Loop-mediated amplification (LAMP) for on-site screening has been proposed as a future POC test for chronic skin ulcers caused by *T. pallidum* and *H. ducreyi* [13, 14]. Recombinase polymerase amplification (RPA), has emerged as a better field-deployable molecular diagnostic tool since it yields readily readable results within a shorter turnaround time (less than 15 min) and less complicated to run at a lower temperature (37 – 42^{o}C) compared to other isothermal techniques such as LAMP [15, 16]. Studies have performed RPA at low resource setting in a mobile suitcase laboratory coupled with a field-friendly DNA extraction techniques for

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effective diagnosis of infectious diseases [17–19]. The technique opens the door to extending the application of DNA amplification in the fieldwork or point-of-need to differentiate between pathogens commonly isolated from chronic skin ulcers [20]. This isothermal nucleic acid amplification technique can be used at all stages of yaws eradication stages especially the follow-up phase, monitor azithromycin resistance as well as identify individuals with *Treponema pallidum* and *H. ducreyi* co-infection in low resource settings. In this study, we have developed a duplex RPA assay for the simultaneous detection of *T. pallidum* and *H. ducreyi* (TPHD RPA) in DPP confirmed yaws patients.

2. Materials and Methods

2.1 Clinical samples

Samples were obtained from an ongoing skin neglected tropical diseases (NTDs) project (Yaws, Leprosy and Buruli ulcer) community outreach program being conducted in Wassa Amenfi East (WAE), Upper Denkyira East (UDE), Upper Denkyira West (UDW), Upper West Akyem (UWA), Akwapim North (AN) and Sekyere afram plains (SAP) districts of Ghana. In brief, clinically suspected yaws patients were first screened with the Syphilis Rapid Diagnostic Test (RDT) and if positive, then tested with the Dual Path Platform (DPP) Syphilis Screen-and-Confirm assay according to manufacturer instructions. Swab samples were taken for both ulcers and wet papilloma while scab was removed from dry or closed papilloma from patients who were DPP positive. Samples were placed in a sterile patient ID pre-labeled cryotubes containing 700 µl cell lysis solution (CLS) and transported to Kumasi Center for Collaborative Research into Tropical Medicine (KCCR) and Noguchi Memorial Institute for Medical Research (NMIMR) depending on the proximity of the district to the reference laboratory for further molecular analysis.

This study used samples collected from an ongoing project. For use of archive samples for future research activities, consent from study participants and approval from the Committee for Human Research Ethics and Publication (CHRPE), School of Medical Sciences, Kwame Nkrumah University of Science and Technology was obtained.

2.2 DNA extraction

The Gentra Puregene Tissue Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. Briefly, samples stored in lysis buffer were vortexed for 2 min, swab sticks removed from the solution and then centrifuged for 1 min at 13,000 x g to pellet the cells. Supernatant was carefully discarded. A total of 300 µl of cell lysis solution (CLS) was added to the pellet after which 15 µl of lysozyme was added and mixed by inverting 25 times. It was then incubated at 37°C for 30 mins and at 80°C for 5 mins to lyse the cells. Proteins were precipitated by adding 100 µl of protein precipitation solution (PPS) to the sample, vortexed 20 secs and centrifuge at high pellet. Supernatant was transferred into a pre-labelled 1.5 ml eppendorf tube, containing 300 µl isopropanol and 2 µl glycogen. This mixture was mixed by inverting gently 50 times and then centrifuged for 1 min at high speed to pellet DNA. The supernatant was carefully discarded and 300 µl of 70% ethanol added to wash the DNA and then centrifuged for 1 min. Alcohol supernatant was carefully discarded and DNA air dried for 5-30 mins. The extracted DNA was resuspended in 100µl DNA

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Hydration Solution, vortex for 5 sec and incubated at 65 °C for 1 h. Extracted DNA was stored at –20°C till it was used for duplex *T. pallidum* and *H. ducreyi* RPA (TPHD-RPA) and the multiplex real-time PCR (qPCR).

2.3 *T. pallidum* and *H. ducreyi* multiplex qPCR assay

The multiplex qPCR assay was performed with RealCycler® universal (Progenie molecular, Valencia, Spain) for the detection of the PolA and hgbA specific genes for *T. pallidum* and *H. ducreyi,* respectively, on a Bio-Rad CFX96 system according to manufacturer instructions. Six microliter of DNA template was used. Positive and negative controls were also included. Cycling condition of PCR are as follows one (1) cycle of initial denaturing at 95˚C for 15 minutes followed by 45 cycles of 95˚C (denaturing) for 5 secs, 60˚C (annealing) for 30 secs and 72°C (extension) for 30secs.

2.4 *T. pallidum* and *H. ducreyi* (TPHD) RPA assay

2.4.1 Molecular standard

Molecular DNA standard of 600 bp, made up of 300 bp *T. pallidum PolA* gene (GenBank: U57757.1) and 300 bp of *H. ducreyi hemolytic cytotoxin HhdA* gene (GenBank: U32175.1) was synthesized by GeneArt (Invitrogen, Darmstadt, Germany). The number of DNA molecules per microliter was determined using the equation described previously [21]. A serial dilution between 10^6 and 10^0 of the molecular standard were prepared.

2.4.2 TPHD RPA primers and probes

PolA and *HhdA* genes of *T. pallidum* (TP) and *H. ducreyi* (HD), respectively, were used as target sequences for RPA detection. RPA primers and probes were designed in accordance with the TwistDx manufacturer's recommendations (TwistDx Limited, United Kingdom). Three forward primers (FPs), 3 reverse primers (RPs) and one exo probe (P) each for *T. pallidum* (TP) and *H. ducreyi* (HD) (Additional file 1: Table 1) were design by using the Molecular Evolution Genetics Analysis version 7 software (Mega7). In silico BLAST analysis of these oligonucleotide demonstrated high specificity to the *T. pallidum* and *H. ducreyi*. All the primers were produced by Eurofins Genomics (Ebersberg, Germany) and the exo probe by TibMolBiol (Berlin, Germany).

2.4.3 TPHD duplex RPA assay

Preliminary screening of 3 forward primers, 3 reverse primers and one exo probe combinations were tested with 10^5 molecules/10 µl of molecular standard for TP and HD separately (singleplex) using the TwistAmp Exo kits according to the manufacturer's instructions in a final reaction volume of 50 µl (TwistDX, Cambridge UK). Reaction tubes were incubated in a tubescanner (Twista, TwistDx, Cambridge, UK) for 15 min at 42 °C. The fluorescence signals were measured at 20 s intervals. A combined threshold and first derivative analysis were used for signal interpretation. The best primer and probe combination for *T. pallidum* and *H. ducreyi* were later produced as mix for subsequent duplex RPA assays using TwistAmp Exo kits with slight modification to manufacturer's instructions. The reaction was 50 µl containing 2.5 µl each of TP and HD 10 µM primer/probe mix, 29.5 µl rehydration buffer, 8.0 µl DNase-free water, 2.5 µl of 280 mM Magnesium Acetate (MgOAc). Five microliters of DNA template was used.

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2.4.4 Analytical sensitivity and specificity of Duplex TPHD RPA assay

The sensitivity of the RPA assays was evaluated using as serial dilation (10^6 to 10^0 copies/ µl) of TP/HD DNA molecular standard which was tested eight times in triplicates. The threshold time verse log of detected molecules was plotted, and a semi log regression was calculated using PRISM 8.0 software (GraphPad Software, San Diego, CA). The specificity of both singleplex and duplex RPA assays was evaluated using pathogen genomic DNA as template.

2.4.5 Clinical performance of TPHD RPA assay

The clinical performance of the TPHD RPA assay was assessed with 49 DPP positive yaws suspected samples at KCCR. The results of the RPA assays were compared with *T. pallidum* and *H. ducreyi* multiplex qPCR assay.

Statistical Analysis

All data were entered in Microsoft Excel 2016 while GraphPad Prism v.8 (GraphPad software, San Diego, USA) was used for data analysis and drawing graphs. The limit of detection (LOC) of both PCR and RPA were determined using probit regression analysis (GraphPad Prism v.8). General descriptive information of patients recruited such as percentages, frequency, median nd interquartile ranges were obtained using descriptive statistics. Contingency tables were used to determine the sensitivity, specificity and the predictive values of the TPHD-RPA assay using qPCR results as the gold standard.

3. Results

3.1 Analytical sensitivity and specificity of duplex TPHD RPA assay

The nine oligonucleotide combinations for each assay were screened with the respective molecular DNA standard. Only primers FP3 + RP2 for TP and FP3 + RP1 for HD (Table 1) were able to amplify down to 10 DNA molecules/reaction (Fig. 1a), while the other combinations were not successful.

Table 1: RPA Primer and probe for *T. pallidum* and *H. ducreyi* detection

Name	Sequence	Amplicon size
TP_RPA_FP	GTAACACTAATGTCGAGACTGAAAAGGAGTGC	144 bp
TP_RPA_RP	GAATGATGAGACGCTCACACTTGTTATGC	
TP_RPA_P1	GTATCTGCATCTGCTGTGCAGGATCCGGCA (XT-iBHQ2) (X-dSpacer)(fT-ROX)GTCCAAGCTGTCATG-PH	
Hd_RPA_FP	TACTCAGGCAACGGATACCCAACATGCA	169 bp
Hd_RPA_RP	GAGGTAAATCAGGCTGTTACAGGTCATTTA	
Hd_RPA_P1	TACGCCTAAATCGTTAACTGCGGGATTAGG (XT-iBHQ2) (X-dSpacer)(fT-FAM)AGATGGCCATGGTAG-PH	

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Using the best primer and probe combination, we were able to achieve exponential amplification of *T. pallidum Pol A* gene *and H. ducreyi hemolytic cytotoxin HhdA* gene. The start points for the fluorescence signal for both singleplex RPA assays for 10^6 to 10^3 copies of DNA molecular standard were 5-6 minutes, 6-7 minutes for 10^2 copies, and 8-9 minutes for 10 copies (Figure 1). One DNA molecular copy produced variable results for HD RPA assay.

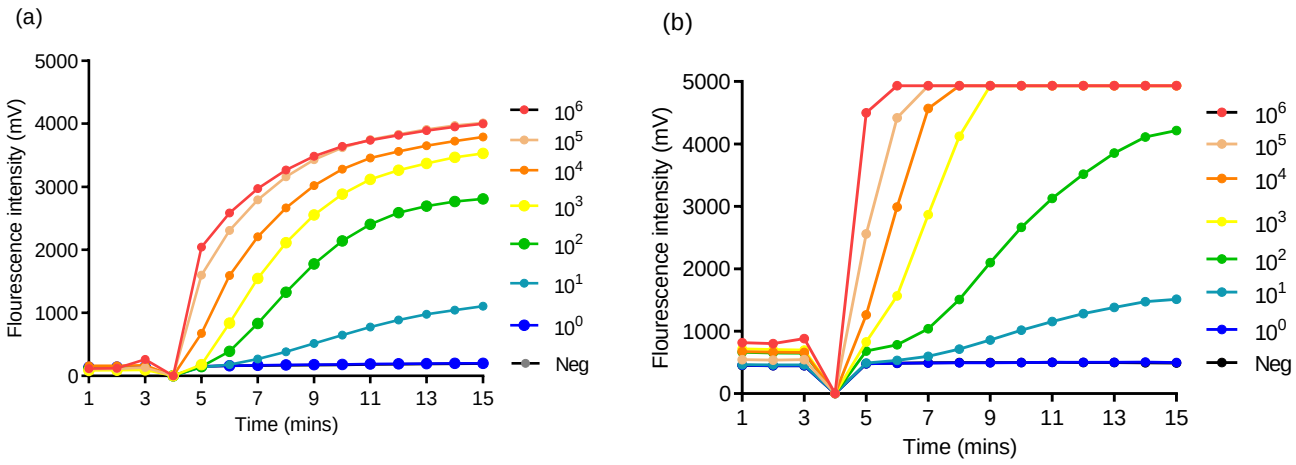

Figure 1: Sensitivity analysis of the singleplex RPA assay using synthetic molecular DNA standard. The assay showed a sensitivity of 10 copies/reaction in *T. pallidum* and *H. ducreyi.* (a) shows the sensitivity test for *T. pallidum*. (b) shows the sensitivity test for *H. ducreyi*.

Using probit analysis, the limit of detection of the singleplex RPA assay at 95% probability was 11 and 6 copies of DNA molecular standard for *T. pallidum* and *H. ducreyi* (Figure 2). The assay showed no cross-reactivity when tested with a panel of 29 genomic DNA of bacterial and parasite species (Table 2).

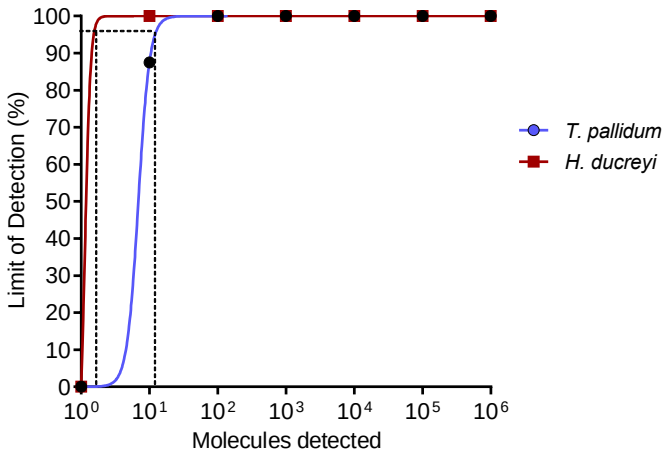

Figure 2: Probit regression analysis. The limit of detection at 95% probability is 11 and 6 DNA molecules for *T. pallidum* and *H. ducreyi*, respectively.

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Table 2: List of bacteria and parasite species and strains used for determining the cross-reactivity of the duplex RPA assay

Name of Bacteria	Source	TPHD RPA
Nocardia ssp.	DSMZ ID: 43757	-
Enterococcus faecalis	DSMZ ID: 1103	-
Streptococcus agalactiae	DSMZ ID: 2134	-
Listeria monocytogenes	DSMZ ID: 15675	-
Staphylococcus aureus	DSMZ ID: 799	-
E. coli	DSMZ ID: 30083	-
Pseudomonas aeruginosa	DSMZ ID: 939	-
Clostridium perfringens	DSMZ ID: 756	-
Plasmodium falciparum	University of Ibadan, Nigeria	-
Leishmania major	American Type Culture Collection, Manassas, USA	-
Leishmania donovani		-
Leishmania aethiopica		-
Leishmania infantum		-
Rickettsia africae	BNITM Hamburg, Germany	-
Rickettsia rickettsia		-
Rickettsia conorii		-
Rickettsia Helvetica	Bundeswehr, Muinch, Germany	-
Mycobacterium marinum	DSMZ ID: 44344	-
Mycabacterium smegmatis	DSMZ ID: 43756	-
Mycobacterium avium ssp. Avium	DSMZ ID: 44156	-
Mycobacterium avium ssp. silvaticum	DSMZ ID: 44175	-
Mycobacterium intracellulare	DSMZ ID: 43223	-
Mycobacterium fortuitum ssp. Fortuitum	DSMZ ID: 46621	-
Mycobacterium gordonae	DSMZ ID: 43213	-
Mycobacterium kansasii	DSMZ ID: 44162	-
Mycobacterium phlei	DSMZ ID: 43239	-
Mycobacterium avium paratuberculosis	ATCC 19638	-
Rhodococcus equi	ATCC 25729	-
Streptococcus uberis	DSMZ ID: 20569	-

3.2 Clinical performance of Single and Duplex TPHD RPA assay

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Table 3 gives a summary of the demographics and yaws-like lesion presented by participants. The median age of participants was 10 years with ulcer being the most (71%) clinical form confirmed positive for yaws by DPP test. Of the forty-nine DDP positive results, 10 were identified as *T. pallidum* lesions, 14 as *H. ducreyi* lesions and 7 lesions coinfected with *T. pallidum* and *H. ducreyi.*

Table 2: Demographic and Yaws clinical presentation of participants

Characteristic	**Frequency N (%)**
Gender	
Male	31 (63)
Female	18 (37)
Age (years)	
Median (IQR)	10 (8-14)
Clinical form	
Ulcer	35(71)
Papilloma	12 (25)
Ulcer and papilloma	2 (4)

The diagnostic performance of duplex RPA assay was evaluated with forty-nine DPP positive samples. Ten (20 %) samples were identified as infected with *T. pallidum*, 14 (29 %) samples infected with *H. ducreyi* lesions, 7 (14%) samples were co-infected with *T. pallidum* and *H. ducreyi* and 18 (37%) negative by qPCR. The duplex RPA assay also correctly identified all samples containing only one single pathogen and 5 (10%) samples being coinfected. Two (4%) coinfected samples were either positive for either *T. pallidum* or *H. ducreyi* and not both. Using qPCR as the gold standard test, the diagnostic sensitivity of the duplex TPHD-RPA assay for *T. pallidum* and *H. ducreyi* was 94 % and 95%, respectively (Table 3). Kappa coefficients (κ), ranging from 0.95 - 1 for the detection of *T. pallidum* and *H. ducreyi* demonstrate an excellent diagnostic agreement between qPCR and our developed duplex TPHD-RPA**.** Additionally, excellent agreement between qPCR and TPHD-RPA (κ = 0.9) was demonstrated for lesions coinfected with both pathogens. All 18 negative qPCR samples were negative by TPHD-RPA assay demonstrating 100% specificity.

Table 4: Diagnostic performance of duplex TPHD-RPA assay as compared to qPCR

Characteristics	**sample size**	***T. pallidum***	***H. ducreyi***
Total samples	49		
No. positive	31	16	20
Sensitivity, % (95% CI)		94 (73-100)	95 (77-100)
Specificity, % (95% CI)		100 (89-100)	100 (88-100)

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PPV, % (95% Cl)		100 (81-100)	100 (84-100)
NPV, % (95% CI)		97 (85-100)	97 (83-100)
Samples containing single pathogen*			
Total samples	42		
No. positive	24	10	14
Sensitivity, % (95% CI)		100 (72-100)	100 (78-100)
Specificity, % (95% CI)		100 (89-100)	100 (88-100)
PPV, % (95% Cl)		100 (72-100)	100 (78-100)
NPV, % (95% CI)		100 (89-100)	100 (88-100)
Samples containing both pathogens*			
Total samples	49		
No. positive	7	6	6
Sensitivity, % (95% CI)		86 (49-100)	86 (49-100)
Specificity, % (95% CI)		100(92-100)	100(92-100)
PPV, % (95% Cl)		100 (61-100)	100 (61-100)
NPV, % (95% CI)		98 (88-100)	98 (88-100)

4. Discussion

Eradication of yaws is a major priority by WHO with the detection of *H. ducreyi* an essential component in the management of yaws patients. In yaws endemic communities, clinical diagnosis combined with serological testing is the sole means of diagnosing suspected yaws cases. Molecular assays especially nucleic acid amplification techniques can help overcome the limitations of serological tests and hence are needed in the WHO yaws eradication strategy [7, 8, 10, 11].

In this study, we developed a real-time recombinase polymerase assay for the simultaneous detection of *T. pallidum* and *H. ducreyi* (TPHD-RPA). The developed TPHD-RPA assay was highly specific to the target organisms with LOD of 11 and 6 copies of DNA for *T. pallidum* and *H. ducreyi* respectively. This analytic sensitivity is comparable to multiplex qPCR whose sensitivity is 100 and 1 copy for *T. pallidum* and *H. ducreyi* respectively (RealCycler® universal TPHD-U TPHD-G protocol V.4). The diagnostic performance of the duplex TPHD assay was better than the singleplex RPA assay demonstrating a much better means of diagnosing of yaws and *H. ducreyi* cases. The turnaround time of 15 mins for the developed RPA assays suggest that the RT-TPHD RPA could provide rapid molecular diagnosis of *T. pallidum* and *H. ducreyi* lesions and also be very essential in areas where co-infection has been reported [9, 22].

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Polymerase chain reaction provides high sensitivity and specificity for diagnosing yaws and *H. ducreyi* lesions [7, 8, 11]. However, operating this technique at the point of care is very challenging because of its requirement for sophisticated laboratories equipped with expensive equipment such as a thermocycler [12] which can cost more than 15 times as much as isothermal fluorometers which enable the performance RPA. RPA performance requires less costly equipment and can be performed in less resource settings in a mobile suitcase laboratory. [17, 23]. Due to PCR being limited to reference laboratories, TPHD-RPA could be an alternative molecular diagnostic tool which can be essential in the yaws eradication strategy.

Recently, the use of Loop-mediated amplification (LAMP) for on-site screening has been proposed as a future point of care test for chronic skin ulcers caused by *T. pallidum* and *H. ducreyi* (TPHD-LAMP) [13, 14]. This TPHD-LAMP had a limit of detection of 300-600 DNA copies with a diagnostic performance of 85-92 % sensitivity and 85-96% specificity [14]. In contrast to our study, the TPHD-RPA assay recorded 86-100 % sensitivity and 100% specificity. These diagnostic performances of both TPHD-RPA and TPHD-LAMP suggest the potential use of these amplification techniques as an alternative to PCR for confirming clinically suspected yaws cases. However, RPA has an added advantage of rapidly amplifying nucleic acid target at 39°C - 42 °C within a short test time of 15 min compared to LAMP which requires a higher temperature of 60°C with a turnaround time of an hour [13, 14]. Also, all critical RPA reagents are lyophilized into a single pellet which ensures the stability of reagents at room temperature for at least six months, reduces contamination, facilitates easy transportation of reagents and simplifies RPA applicability in low resource settings [24, 25].

This study used a small number of samples to evaluate the developed duplex TPHD RPA and hence further evaluation, as well as a multinational evaluation, is needed. This would help ascertain the diagnostic performance and the role this assay could play in support of national yaws eradication programs when deployed in different yaws-endemic countries. Prior to such evaluation program, a field-friendly DNA extraction technique such as GenoLyse and SpeedXtract [26–28] needs to be evaluated with the duplex TPHD-RPA assay.

5. Conclusions

We have developed a simple novel TPHD-RPA assay which can enable rapid differential detection of *T. pallidum* and *H. ducreyi* in Yaws-like lesions. The diagnostic performance of the TPHD-RPA suggests its potential to increase access to molecular diagnosis of yaws, especially in low resource yaws endemic communities. Also, this test could support all yaws eradication programs by ensuring effective diagnosis as well as enable monitoring of eradication efforts success or failure and planning of follow-up interventions at the community level.

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Amanor I.B., Böhlken-Fascher S., Kissenkötter J., Addo K.K., Wahed A.A.E., and Phillips R.O.; visualization, Frimpong M. and Wahed A.A.E.; supervision, Addo K.K., and Phillips R.O.; project administration, Frimpong M. Addo K.K., and Phillips R.O.; funding acquisition, Frimpong M. Addo K.K., Wahed A.A.E., and Phillips R.O. All authors have read and agreed to the published version of the manuscript.

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