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Development of field-applicable tests for rapid and sensitive detection of *Candidatus* Phytoplasma oryzae

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ABSTRACT

Napier grass Stunt Disease (NSD) is a severe disease of Napier grass (*Pennisetum purpureum*) in Eastern Africa, caused by the leafhopper-transmitted bacterium *Candidatus* Phytoplasma oryzae. The pathogen severely impairs the growth of Napier grass, the major fodder for dairy cattle in Eastern Africa. NSD is associated with biomass losses of up to 70% of infected plants.

Diagnosis of NSD is done by nested PCR targeting the phytoplasma DNA, which is difficult to perform in developing countries with little infrastructure. We report the development of an easy to use, rapid, sensitive and specific molecular assay for field diagnosis of NSD. The procedure is based on recombinase polymerase amplification and targets the *imp* gene encoding a pathogen-specific immunodominant membrane protein. Therefore we followed a two-step process. First we developed an isothermal DNA amplification method for real time fluorescence application and then transferred this assay to a lateral flow format. The limit of detection for both procedures was estimated to be 10 organisms. We simplified the template preparation procedure by using freshly squeezed phloem sap from Napier grass. Additionally, we developed a laboratory serological assay with the potential to be converted to a lateral flow assay. Two murine monoclonal antibodies with high affinity and specificity to the immunodominant membrane protein IMP of *Candidatus* Phytoplasma oryzae were generated. Both antibodies specifically reacted with the denatured or native 17 kDa IMP protein. In dot blot experiments of extracts from infected plant, phytoplasmas were detected in as little as 12,5 μg of fresh plant material.

1. Introduction

Phytoplasmas are insect transmitted phloem-limited bacterial plant pathogens parasitizing sieve tubes and causing profound disturbance of growth and productivity of numerous cereal, vegetable and fruit crops, as well as ornamental plants and trees worldwide [1]. In East Africa, Napier grass (*Pennisetum purpureum*), a fast-growing and high-yielding perennial grass native to Africa is affected by phytoplasmas in many regions. Infected grass exhibits severe yellowing and stunted growth prompting the disease name, Napier grass Stunt Disease (NSD). The disease causes up to 70% loss in biomass per infected plant, decimating the economic livelihoods of thousands of smallholder farmers who rely on Napier grass as feed for their dairy animals, which generate their income and provide their food. NSD was first reported in Kenya in 2004 [2], and

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later in Ethiopia [3], Uganda and Tanzania [4]. The phytoplasmas associated with NSD in Kenya, Uganda and Tanzania belong to the species *Candidatus* Phytoplasma oryzae (*Ca*. P. oryzae) [2,4] while the pathogen in Ethiopia is a member of the *Candidatus* Phytoplasma pruni species [3]. In Kenya *Maiestas banda* Kramer, a phloem-sucking leafhopper has been identified as a vector of Napier stunt phytoplasma [5], although other as yet undescribed vectors might exist. The common practice of sharing and trading cuttings and root splits of Napier grass by farmers as planting materials accelerates the spread of NSD. Wild grasses offer an alternative host for the Napier stunt phytoplasma [6] and the phytoplasma can potentially be transmitted to graminaceous cereals such as maize and sorghum, which are the most important staple food crops in Eastern Africa [7].

Phytoplasmas are unculturable *in vitro* and routine laboratory detection depends on molecular and serological methods. Molecular methods are mostly based on PCR assays using 16S ribosomal genes as the principal target for detection and characterization of phytoplasma species [1,8]. Varying primer combinations have been developed for universal [9], generic [10,11], and species-specific [12,13] detection of phytoplasmas in plant or insect samples. Routine detection of NSD phytoplasma is performed by amplification of the 16S rDNA in a nested PCR assay consisting of two subsequent PCRs [9]. This approach has been applied in very few laboratories across Eastern Africa to detect NSD phytoplasma in Napier grass [2–4], insect vectors [14] and wild grasses [6]. Nielsen et al. [4] used a real time Taqman PCR assay developed earlier [15] to simultaneously detect and quantify the NSD phytoplasma while using the plant 18S rDNA as internal control. Although PCR-based assays are sensitive, these methods are of restricted use in low and middle-income countries with poor laboratory infrastructure and limited resources as well as unreliable power supply. In addition, the current NSD phytoplasma diagnostics are based on the 16S rDNA gene, which is highly conserved and inadvertently raises the risk of false positives due to cross-amplification of related phytoplasmas and non-target bacteria with similar sequences, reducing the specificity of the tests [11]. Post-PCR procedures like RFLP analysis and DNA sequencing are required to verify the PCR amplicons unequivocally increasing the overall cost and expenditure of time for diagnosis.

Recent studies have demonstrated the feasibility of isothermal amplification technologies like loop-mediated amplification assay (LAMP) as viable alternatives for on-farm detection of phytoplasmas [16–18] but LAMP is highly susceptible to false-positive results. The lack of multiplexing capability and the complex primer design are additional disadvantages of the method. Although a LAMP assay for NSD detection based on 16S rDNA was previously developed [19], the specificity of the assay was low, data on the sensitivity of the assay were absent, and no field evaluation of the method was conducted. The recombinase polymerase amplification (RPA) technique is becoming a popular molecular tool for the rapid, sensitive and cost-effective detection of pathogens. It has been applied for the detection of plum pox virus among others and for the detection of methicillin-resistant *Staphylococcus aureus* [20], *Mycobacterium tuberculosis* [21], HIV-1 [22] and *Mycoplasma capricolum* subsp. *capripneumoniae* [23]. RPA is a versatile DNA amplification technique and can be combined with a range of detection procedures. Besides a real time assay based on fluorophore-labeled probes comparable to the Taqman real-time assays, amplification products can be visualized after incorporation of biotin-labeled primers with lateral flow devices. RPA and lateral flow assays (RPA-LF) have been developed for *Plasmodium falciparum* [24] and *Giardia duodenalis* [25]. Lateral flow assays are easy to use and therefore appropriate for resource-poor settings and nontechnical staff.

Serological detection methods are widely applied in plant pathology and belong to the standard repertoire of plant pathology laboratories and plant protection facilities. The diagnostic procedures are specific, easy to perform and cost efficient. Although a number of polyclonal antisera and monoclonal antibodies have been generated in the past against several phytoplasmas and varying epitopes, their employment for routine diagnosis is low and mostly restricted to the immunohistological localization of the pathogen [26]. The most widely used antigens for the generation of specific antibodies are the immunodominant membrane proteins (IMPs) [27–31]. IMPs are present in large numbers in the phytoplasma membrane and represent promising targets for the development of detection assays. Serological detection procedures are not available for NSD phytoplasma at present.

In this study, we developed an urgently needed field-applicable molecular diagnostic assay and a serological assay based on the immunodominant membrane protein gene (*imp*) and its respective product (IMP). Additionally, we compared the recombinase polymerase amplification (RPA) technology with the existing PCR-based assays. For the serological detection of IMP, high affinity monoclonal antibodies were generated and tested in immunoblot and enzyme-linked immunosorbent assay (ELISA) experiments for performance. Protocols for sample preparation from Napier grass and insects were also simplified for easy and rapid use in the field.

2. Materials and methods

2.1. Samples used in this study

About 200 leaf and stem cuttings of Napier grass (*Pennisetum purpureum*) cultivars were collected in February and March 2014 from farms in Kisumu, Vihiga, Butere, Busia, Teso, Bungoma, Siaya and Mbita districts of Western Kenya. They were screened for NSD infection and a subset of 96 samples representative of the different geographical origins were used for subsequent analyses (Table S1). A set of ten Napier grass plants maintained in *icipe*'s experimental plots at Thomas Odhiambo Campus in Mbita Point were used for the development of molecular and serological assays. Napier grass specimens free of any phytoplasma infection were accessed from healthy plants kept under insect-proof conditions in a greenhouse in *icipe*'s field station in Mbita Point and from the International Livestock Research Institute campus, an environment with no history of NSD.

Leaves of 17 periwinkle plants (*Catharanthus roseus*) infected with 15 different species of phytoplasmas available at the Julius Kühn Institute were also sampled for specificity testing (Table 1). In addition, two DNA extracts of *Candidatus* Phytoplasma cynodontis (CN, Kenya, 16Sr-XIV) were used for specificity testing.

Finally, 300 leafhoppers (*Maiestas banda*) fed on infected Napier grass in insectaries at *icipe* in Mbita Point were also included in the study. Upon collection, plant and insect samples were processed immediately or kept at −20 °C until DNA extraction.

2.2. DNA extraction

Genomic DNA was extracted from plants and insects using a cetyltrimethylammonium bromide (CTAB) method [32]. Briefly, 1 g of Napier grass, periwinkle leaves or two insects were placed in an extraction bag (Bioreba, Switzerland) and homogenized with a steel-ball roller in one ml of CTAB buffer. The homogenate was transferred to a microcentrifuge tube and extracted with an equal volume of chloroform:isoamyl alcohol (24:1). The aqueous and organic phases were separated by centrifugation. DNA was precipitated from the aqueous phase by adding an equal volume of isopropanol. The pellet was washed twice with 70% ethanol, air-

Table 1
Phytoplasma species examined for assessing specificity of the RPA and serological NSD phytoplasma detection assays.

Common name	*Candidatus* Phytoplasma species	16SrDNA subgroup
American aster yellows	*Candidatus* Phytoplasma asteris	16SrI
Lime witches' broom	*Candidatus* Phytoplasma aurantifolia	16SrII-B
Faba bean phyllody	na	16SrII-C
Peach yellow leaf roll	*Candidatus* Phytoplasma pruni	16SrIII-A
Green valley strain of western X	*Candidatus* Phytoplasma pruni	16SrIII-A
Rubus stunt	*Candidatus* Phytoplasma rubi	16SrV
Grapevine flavescence doree	*Candidatus* Phytoplasma vitis	16SrV-C
Ash yellows	*Candidatus* Phytoplasma fraxini	16SrVII-A
Almond lethal disease	*Candidatus* Phytoplasma phoenicium	16SrIX-D
Apple proliferation (strain AP)	*Candidatus* Phytoplasma mali	16SrX-A
Apple proliferation (strain AT)	*Candidatus* Phytoplasma mali	16SrX-A
Pear decline	*Candidatus* Phytoplasma pyri	16SrX-C
European stone fruit yellows	*Candidatus* Phytoplasma prunorum	16SrX-F
Rice yellow dwarf	*Candidatus* Phytoplasma oryzae	16SrXI
Australian grapevine yellows	*Candidatus* Phytoplasma australiense	16SrXII
Grapevine yellows	*Candidatus* Phytoplasma solani	16SrXII
Bermuda grass white leaf (Kisumu)	*Candidatus* Phytoplasma cynodontis	16SrXIV
Bermuda grasswhite leaf (Mbita)	*Candidatus* Phytoplasma cynodontis	16SrXIV
Hibiscus witches' broom	*Candidatus* Phytoplasma braziliense	16SrXV
Bovine Mycoplasma	*Mycoplasma mycoides* subps. *mycoides*	N/A

N/A: not assigned to a *Candidatus* Phytoplasma species or 16Sr group.

dried and reconstituted in 50 μl of sterile distilled water. DNA concentrations of sample extracts were determined by spectrophotometry on the ND-2000 instrument (ThermoFischer Scientific) and Qubit® dsDNA HS Assay Kit (ThermoFisher Scientific). The DNA concentration for all samples was adjusted to 50 ng/μl and stored at −20 °C for subsequent analysis.

2.3. PCR-amplification, sequencing and quantification of phytoplasmas in plants and insects

The presence of NSD phytoplasma in total DNA derived from plant samples and insect pools was confirmed by nested PCR targeting the 16S rDNA gene as previously described [5] (Table S2). The nested PCR products were analyzed by agarose gel electrophoresis for the expected band size of 780bp.To confirm the amplified phytoplasmas belonged to the species *Ca*. P. oryzae, we selected 9 NSD accessions showing strong PCR signals and sequenced the first round PCR products using primers P1, P6, NapF and NapR (Table S2). The sequences were deposited under NCBI accession numbers MF281659-MF281667.

The titre of phytoplasmas in ten samples of infected Napier grass (plants maintained in Mbita, Kenya) was determined using real time quantitative PCR (Q-PCR) based on 16S rDNA, as described by Christensen et al. [15], with a few modifications. Briefly, the total reaction mixture of 25 μl comprised 1x DreamTaq mastermix (Thermoscientific, USA), 300 nM of each forward and reverse primer and 75 nM of probes targeting phytoplasma 16S rDNA. In the case of insect samples, quantification was done as previously described [33]. Each reaction was run in triplicate. After 40 amplification cycles, the concentration of phytoplasmas in the test samples was inferred from titration curves of serial dilutions of a plasmid DNA standard containing an apple proliferation phytoplasma 16S rDNA gene.

2.4. Characterization and sequence diversity of the Ca. Phytoplasma oryzae imp gene

To characterize and sequence the immunodominant membrane protein gene (*imp*) of *Ca.* P. oryzae, a nested PCR was designed. A multiple alignment of a 5 kb region reported to contain the immunodominant protein gene [30] was generated from seven phytoplasma species, including rice yellow dwarf strain RYD (GenBank: AB469012), Tsuwabuki witches' broom strain TWB (GenBank: AB469014), Korean potato witches' broom strain PWBK (GenBank: AB469013), porcelain vine witches' broom strain PvWB (GenBank: AB469011), Paulownia witches' broom strain PaWB (GenBank: AB469010), mulberry dwarf MD strain (GenBank: AB469009), onion yellows strains OY-NIM (GenBank: AB469008) and OY-W (GenBank: AB469007). Based on the alignment, one set of degenerate outer (f_long_imp/r_long_imp) and one pair of specific inner (imp_F1/imp-R1) primers were designed (Table S2). Two μl (100 ng) of DNA from phytoplasma-positive Napier grass were used for each reaction. A DNA sample from healthy Napier grass was included as a control. Reaction conditions were as previously reported [30]. The secondary PCR amplicons were cloned into the pGEM-T vector (Qiagen), transformed into competent cells, plasmid DNA isolated and sequenced.

The sequences of the *imp* gene from 26 infected Napier grasses collected from farmer's fields (Table S1) were aligned using Clustal Omega [34] and a phylogenetic tree was constructed based on the Maximum likelihood algorithm in PhyML 3.0 [35].

For membrane topology prediction the Constrained Consensus TOPology Prediction Server (http://cctop.enzim.ttk.mta.hu) was used which comprises the analysis of 11 prediction programs (HMMTOP, MemBrain, MemSat, Octopus, Philius, Phobius, Pro, Prodiv, Scampi, ScampiMsa and TMHMM).

2.5. Development of a recombinase polymerase amplification (RPA)-based detection assay targeting the imp gene of Ca. Phytoplasma oryzae

2.5.1. Design and optimization of RPA primers and probes

Primers and probes were designed based on the full-length DNA sequence of the *imp* gene deposited under Genbank accession KU820961 and 26 full-length *imp* sequences of strains belonging to the same species determined in this study. Five sets of overlapping forward and reverse primers (Table S2) were designed according to the manufacturer's instruction (TwistDX) in Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). Probes for the real-time RPA assay (exo-probe) and the endpoint lateral flow assay (LF probe) were designed manually following the guidelines provided in the TwistDx manuals. Primers were synthesized by Macrogen (Korea) and probes by Biosearch technologies (Petaluma, CA).

The Basic RPA assay (TwistAmp Basic assay) was conducted to

compare the performance of the five primer sets in amplifying *Ca.* P. oryzae *imp* gene according to the manufacturer's guidelines (TwistDx, UK). Briefly, in 50 μl reactions, 29.5 μl of the supplied buffer, 480 nM of each forward and reverse primer, 5 μl (250 ng) of DNA, 2 μl of PCR-grade water and 14 mM of magnesium acetate were added to the enzyme pellets. The reactions were incubated at 39 °C for 20 min in a T-16 isothermal instrument (TwistDx, UK), after which the products were cleaned up on PCR purification columns (Qiagen, USA) and visualized by electrophoresis on a 2% v/w agarose gel stained with ethidium bromide.

Real time RPA assays (TwistAmp®exo assay) were performed according to the standard guidelines (TwistDx, UK). The tests were performed in a 50 μl reaction volume comprising the enzyme pellets rehydrated with 29.5 μl of the supplied buffer, 420 nM of the forward and reverse primers, 120 nM of the appropriate probe (exo probe or LF probe) and 5 μl of total DNA. RPA reactions were initiated by the addition of 14 mM of magnesium acetate. The reaction tubes were incubated at a constant temperature of 39 °C in the T-16 isothermal instrument (TwistDx, UK) for 20 min. Real-time RPA reactions were monitored by observation of amplification curves on the T-16 instrument. Reactions were considered positive when the fluorescence passes the software generated threshold line.

2.5.2. Determination of the specificity of RPA primer and probes

The cross-reactivity of the *imp*-RPA assay was tested against 15 other phytoplasma species (Table 1). We also included one sample of another mollicute species, *Mycoplasma mycoides* subsp. m*ycoides* strain Afade. The DNA concentration and running parameters for real-time RPA reactions (TwistAmp®exo) were as described above. The reactions were monitored by real time observation of amplification curves on the T-16 instrument.

2.5.3. Determination of the sensitivity of the RPA reaction

The sensitivity of the RPA assays was determined using a calibration curve that was established employing the full-length *imp* gene sequence of *Ca.* P. oryzae (Genbank accession KU820961) inserted into pGEM-T (Promega) and used as DNA calibration standard. The number of *imp* copies in the plasmid preparation was determined with respect to the size of the *imp* gene, the size of the vector backbone and the plasmid DNA quantity. Serial tenfold dilutions of the standard (10^6 copies/μl to 1 copy/μl) were prepared and 1 μl was added to the reactions. The real time RPA assays (TwistAmp®exo) and endpoint lateral flow assays (TwistAmp®nfo) were performed as described above. The effect of mechanical agitation on the efficiency of amplification was evaluated by incubating the reactions at 39 °C for 20 min either with agitation for 4 min followed by further incubation for 16 min, or continuously for 20 min without any agitation.

2.5.4. Direct detection of NSD phytoplasma in phloem sap of Napier grass and crude lysates of leafhoppers

The RPA assay was optimized to detect *Ca.* P. oryzae directly from the phloem sap of Napier grass stems. Briefly, the phloem sap from 10 symptomatic Napier grass samples and a healthy control was obtained by cutting individual grass stems with a pair of scissors that was previously cleaned and disinfected with bleach. The sap was squeezed out using a pair of sterilized pliers and approximately 5 μl of sap was collected into a sterile capillary tube. Insect lysates were prepared by crushing individual insects in 1.5 ml tubes with 20 μl of PCR-grade water using sterile polypropylene homogenizers. No further processing for genomic DNA extraction was undertaken. The crude plant and insect preparations were tested for DNA amplification using 5 μl of undiluted and diluted in sterile distilled water (1:10, 1:50 and 1: 100) samples with real-time RPA assay (TwistAmp®exo).

2.5.5. Transfer of the RPA assay to a field-applicable dipstick platform

Lateral flow assays (TwistAmp®nfo assay) were performed according to the standard guidelines (TwistDx, UK). Products from the lateral flow assays were evaluated by loading 5 μl of the amplicons diluted 1:1 with nuclease-free water on lateral flow cassettes with a biotin capture molecule (BioUSTAR, Hangzhou, China).

2.5.6. Comparative analysis of nested PCR, real-time PCR and RPA methods in detection of NSD phytoplasma

The diagnostic accuracy of the RPA assay developed in this study was evaluated by comparing it with published protocols for nested PCR and real-time PCR for *Ca.* P. oryzae. A set of 66 DNA samples extracted from Napier grass collected in farmer's field (Table S1) was therefore employed. The 16S rDNA nested-PCR was used as the reference assay. Diagnostic sensitivity, specificity, positive predictive values and negative predictive values were calculated according to Faye et al. [36].

2.6. Development of a serological detection assay targeting the IMP protein of Ca. P. Oryzae

2.6.1. Cloning of the imp gene, expression and purification of recombinant IMP

The full-length *imp* gene from *Ca.* P. oryzae strain Mbita1 was PCR amplified and ligated into pGEM-T via added restriction sites *Bam*HI and *Sal*I at the 5'- and 3′ end, respectively. The gene was subcloned into the expression vector pQE30 (Qiagen) and transformed into *Escherichia coli* strain Bl21λDE3. Recombinant IMP was expressed overnight after induction of protein expression using 1 mM IPTG. Bacterial cells were lysed with B-PER™ (Bacterial Protein Extraction Reagent, ThermoScientific). The insoluble recombinant protein was solubilized in 6 M guanidinium-hydrochloride and purified under denaturing conditions via Ni-NTA agarose according to the manufacturer's instructions (Qiagen, The QIAexpressionist 06/2003, protocol 19). The protein eluate was dialyzed at 4 °C overnight against PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.02% Na-azide) to remove urea. Protein expression and all subsequent purification steps were monitored by analyzing aliquots from each manipulation step by polyacrylamide gel electrophoresis followed by Coomassie Blue staining or by immunoblot analysis using anti his-tag antibodies.

2.6.2. Generation of murine monoclonal Ab against recombinant IMP

Two BALB/c mice were immunized four times with the IMP-antigen according to standard procedures. After each boost blood was taken to monitor the specific immune response in ELISA assays against the recombinant protein. B-lymphocytes from the spleen were fused with SP2/0-AG14 myeloma cells and the primary cultures were analyzed for antigen-specific IgG production in ELISA assays. Monoclonal cell lines were derived from the primary culture by limiting dilution and screened again for specific anti-IMP IgG production by ELISA. Preparative IgG production was performed after adaptation of hybridoma lines to serum-free medium. IgGs were purified via affinity chromatography by MEP HyperCell™ Mixed-Mode Chromatography Sorbent (Pall, USA). The antibody concentration was determined by SPR spectroscopy (RAMFc) and antibody integrity was visualized by SDS PAGE analysis.

2.6.3. Screening of hybridoma cell lines for IgG production and detection of IMP protein in protein extracts from plant material using enzyme-linked immunosorbent assays (ELISA)

Hybridoma supernatants were screened for the presence of specific IgGs by ELISA with microtiter plates coated with 0.5 μg IMP per well. After washing off unbound IMP with PBS-Tween20 (PBS, 0,05% PBS), hybridoma supernatants were added to individual wells and incubated for 1.5 h at 37 °C. Non-specifically bound antibodies were removed by washing with PBS-Tween20. Alkaline phosphatase-labeled goat-anti-mouse (GAM^{AP}) IgGs were added as secondary antibodies for 1 h at 37 °C. After washing the plates, N-nitrophenly-phosphate was added for color formation. The enzymatic activity was recorded at 405 nm on a plate reader.

Protein preparations from *Ca.* P. oryzae-infected Napier grass sample Mbita1 (Table S1) and healthy Napier grass were diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% Na-azide; pH9.6) and dilutions thereof were added to ELISA plate wells. Purified IMP-specific monoclonal antibodies were diluted 1:5000 for antigen detection. All subsequent steps were performed as described above.

2.6.4. Protein extraction from plant material and immunoblot assays

Total protein extraction from fresh plant material was performed by two simple extraction procedures. Briefly, plant material (0.5 g) was homogenized in two ml PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.02% Na-azide) or in SDS buffer (190 mM glycine, 25 mM Tris, 1% SDS; pH8.3). One ml of the homogenate was transferred to a microcentrifuge tube and cleared by centrifugation. The supernatant was transferred to a fresh tube and the protein concentration determined by Qubit® protein quantification assays according to the manufactures' instructions (ThermoFisher Scientific). The SDS extract was diluted 1:1 with water before quantification. Aliquots of the extracts were stored at −20 °C until processing.

For polyacrylamide gel electrophoresis (PAGE) protein samples were mixed with SDS loading dye and the proteins were denatured at 98 °C for 5 min. After denaturation proteins were separated in discontinuous 10% or 14% polyacrylamide gels at 15 V/cm for 1.5 h at room temperature in SDS running buffer using a Mini-Protean chamber (Hoefer Scientific). Prestained protein markers were used to assess the molecular weight (ThermoFisher Scientific). PAGE separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes using a blotting transfer system (Mini Trans Blot® Cell, BioRad) at 100 V for 1.5 h at 4 °C. Transfer of the prestained molecular weight marker was indicative of completion of the blotting process. After transfer, the membranes were incubated briefly in blocking solution (2% skimmed milk dissolved in PBS). Then blots were incubated overnight at 4 °C in blocking solution containing the monoclonal antibody diluted 1:1000 or 1:2500 in a head-over-tail shaker. Non-specifically bound antibodies were washed off by three washes with 50 ml of PBS Tween20. Then blots were incubated for 1 h with blocking solution containing GAM^{AP} IgGs diluted 1:10,000 under constant agitation. After three washes blots were overlaid with substrate solution (NBT/BCIP, Serva, Germany) until color reactions were distinct.

2.6.5. Specificity of selected mAb and cross-reaction analysis tested by immunoblot assays

Proteins extracts from leaves of NSD-infected Napier grass, healthy Napier grass (Table S1) and periwinkle plants (*C. roseus*) infected with 16 different phytoplasma species (Table 1) were used for examining the reaction of selected mAbs 28.2 and 37.2 and their species-specificity by immunoblot assays.

2.6.6. Dot blot analysis

PBS-extracted native proteins from infected and healthy plant samples were analyzed by dotting diluted or undiluted protein extracts on nitrocellulose membranes (Amersham). The membranes were dried and incubated with antibody dilutions (up to 1:50,000 in blocking solution) overnight at 4 °C. Washing and detection steps were performed as described above.

3. Results

3.1. Phytoplasma titre in leafhopper and plant samples

The number of *Ca.* P. oryzae copies per gram of plant material was estimated using the calibrated DNA standard after 16S rDNA real-time PCR assay. The titres varied from plant to plant and ranged between 3.82×10^2 to 8.90×10^5 per 100 ng of total extracted DNA. The median value was 1.25×10^4 copies.

From the 300 leafhoppers fed on infected Napier grass, 150 insect pools were tested for the presence of *Ca.* P. oryzae using nested PCR. Forty-three pools were determined to be positive for infection and quantified. The number of copies per μl of extract varied between 1.49×10^3 to 1.13×10^5 per 100 ng of total extracted DNA. The median value was 7.35×10^3 copies.

3.2. Sequence analysis, genetic diversity and phylogenetic comparison of the NDS imp gene

The *imp* gene sequence of 26 NSD phytoplasma accessions collected in different areas in Western Kenya was determined and deposited in Genbank under accession numbers KY449463-KY449488. The sequences comprising 408 bp each were aligned. Only a single nucleotide polymorphism was present in the 26 NSD sequences. The 136 amino acid sequence showed the characteristic domains like other IMPs. The analysis of the membrane topology with the Constrained Consensus TOPology prediction server identified a transmembrane helix at position 15 to 33 relative to the start codon, an upstream part predicted to face inside the cell and the downstream part which faces to the outside.

The rooted phylogenetic tree was built with protein sequences using five *imp* gene sequences from other phytoplasma species as outgroups. The phylogeny confirms that all *Ca.* P. oryzae *imp* sequences cluster together and are well separated from *imp* sequences of other species (results not shown). The NSD *imp* gene sequences were closest to the rice yellow dwarf (RYD) phytoplasma sequence, which is in agreement with previous data based on 16SrDNA classification [37]. The conserved nature of the NSD-*imp* gene sequence and the distinctiveness to other closely related phytoplasma *imp* genes indicated its high suitability for the development of a specific NSD nucleic acid-based detection assay.

3.3. Development of a RPA-based detection assay for NSD phytoplasma

3.3.1. Optimization of RPA primer combinations

Based on the results obtained above, five sets of overlapping primers were designed to amplify the NSD *imp* gene using the RPA assay (Table S2). Combinations of all primer pairs were evaluated to identify the best set for subsequent diagnostic assays. The basic endpoint RPA assay was performed to screen the primer sets across a gradient of 12 temperatures from 36 °C to 43 °C. One DNA template of infected Napier grass was used for all the reactions. The primer set RPA_imp_NSD_Exo_F/R (Table S2) amplified the target region reproducibly across the temperature range producing a specific amplicon of approximately 170 bp, while the remaining primer sets gave multiple amplicons or failed to amplify (results

not shown). The DNA fragment amplified by the primer set above was sequenced and confirmed to be the genuine *imp* fragment. This primer set was therefore selected for downstream RPA reactions.

3.3.2. Specificity of RPA in detection of NSD phytoplasmas

The specificity of the RPA *imp* assay was evaluated for cross-reactivity against 15 phytoplasma species representing eleven phytoplasma 16SrDNA sub-groups and for *Mycoplasma mycoides* ssp. *mycoides* (Table 1). The assay was highly specific to *Ca.* P. oryzae from NSD since no other phytoplasmas gave positive reactions after up to 30 min. All DNA samples extracted from infected Napier grass sampled from different geographical regions in Kenya amplified reproducibly in the assay (Fig. 1).

3.3.3. Sensitivity of RPA in detection of Ca. P. oryzae

To determine the sensitivity of the RPA assay for detection of *Ca.* P. oryzae from Napier grass, varying copy numbers (10^6 copies to 1 copy) of the cloned NSD *imp* gene were subjected to RPA amplification. The limit of detection was assessed with two RPA assays, *imp*-based real-time RPA (TwistAmp®exo assay) and lateral flow RPA (TwistAmp®nfo assay). With the real-time RPA assay, the effect of agitating the tubes 4 min into the reaction (as recommended by the manufacturer) was also evaluated.

The results indicate that the limit of detection varied slightly between the two RPA assay formats. The real-time RPA showed a higher level of sensitivity with a detection of 1–10 gene copies (Fig. 2a and b) whereas the lateral flow RPA had a lower sensitivity with the limit of detection being between 10 and 100 copies (Fig. 2c). The recommended agitation of the reaction 4 min after start accelerated the amplification rate of the real time RPA assay. The threshold fluorescence value was passed 5–8 min after the start, whereas without agitation, the time to reach detectable levels (threshold) ranged between 8 and 14 min (Fig. 2b). Although the assays were run for the maximum duration of 30 min, the plateau phase for agitated reactions was reached by 12 min (720 s) regardless of the template copy number (Fig. 2a and b).

3.3.4. Detection of NSD phytoplasma in phloem sap extracts of infected Napier grass

The previous template preparation protocols included crushing hard leaves and extracting DNA from the plant material. We therefore tested the feasibility of performing RPA reactions on neat phloem sap directly squeezed from the plant. The procedure for sampling sap from Napier grass is depicted in Fig. 3a and b. Ten Napier grass samples with confirmed NSD infection, and one healthy control were examined. The results displayed on Fig. 4a and b showed that the *imp* gene of *Ca.* P. oryzae was amplified in all infected samples, irrespective of the use of DNA or neat phloem sap as template. Positive amplification from phloem sap was slightly slower compared to purified DNA. The time taken to observe detectable fluorescent signals was 3–5 min for DNA templates (Fig. 4a), whereas it took on average 6–16 min to observe detectable signals in phloem sap samples (only one sample required 28 min for detection).

For insects, 30 extracts demonstrated to be positive with nested 16S rDNA PCR were tested using the RPA detection method on crude insect lysate. Here also, we were able to confirm positive amplification without any need for DNA extraction for all extracts

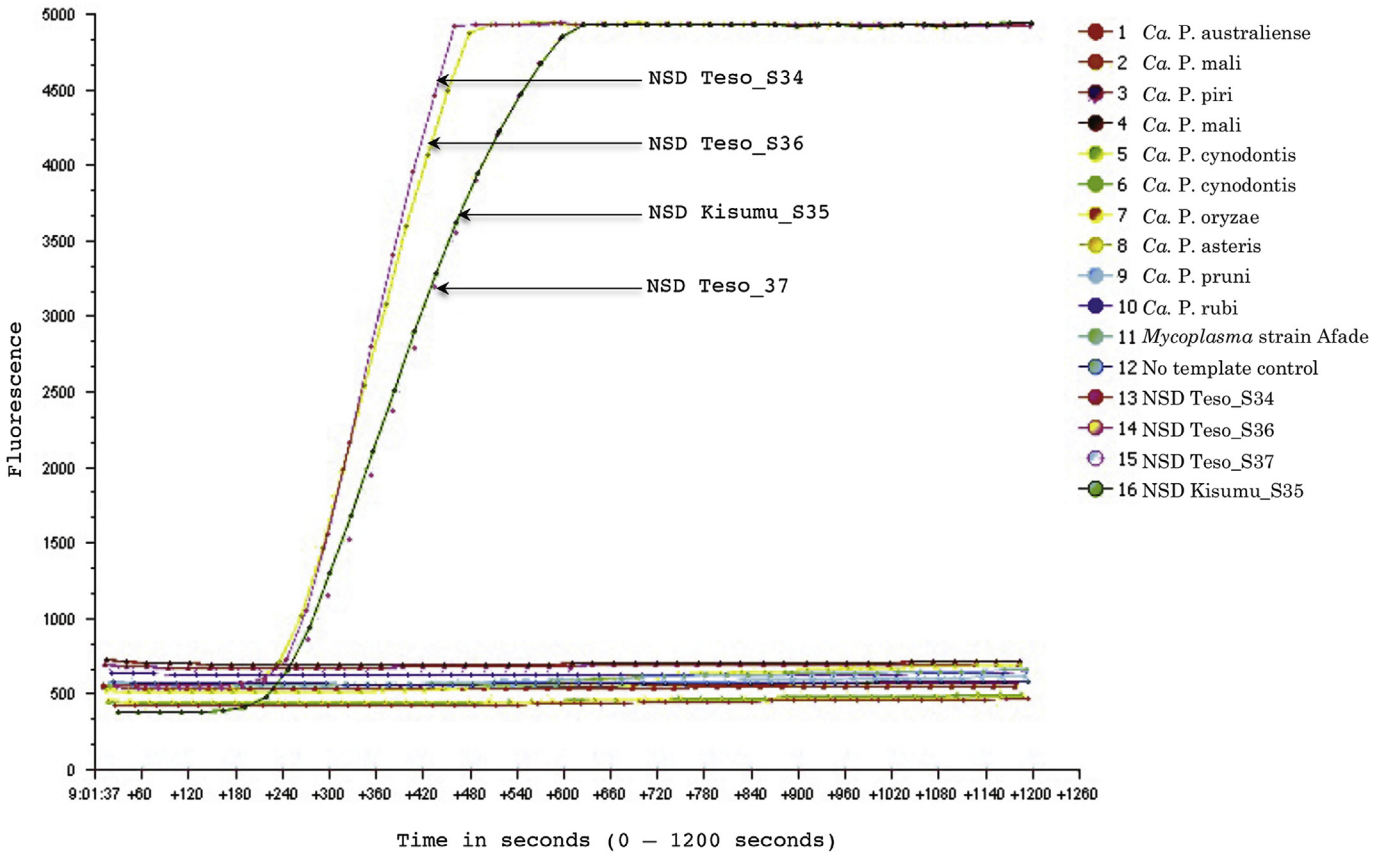

Fig. 1. Specificity analysis of the recombinase polymerase amplification (RPA) assay for NSD phytoplasma. The NSD RPA assay was assessed against other phytoplasma strains to determine cross-reactivity. 100 ng of genomic DNA of each sample were incubated with RPA reagents at a constant temperature of 39 °C for 20 min and amplification observed on a portable fluorometer.

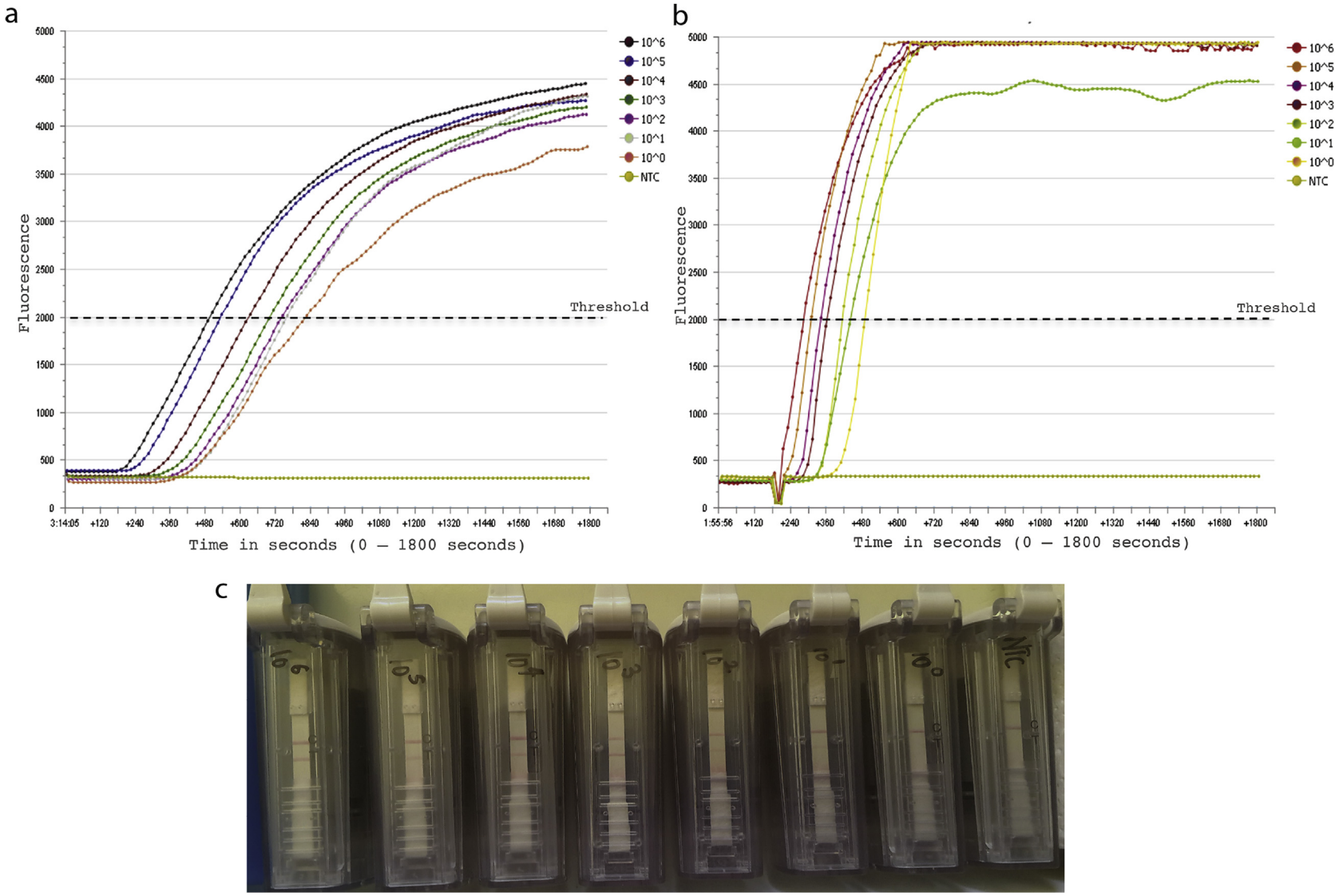

Fig. 2. Sensitivity of the real-time recombinase polymerase amplification (RPA) assay for detection of the *imp* gene of Napier grass stunt phytoplasma (NSD *imp*). a: amplification of the DNA standards without agitation; b: depicts amplification with brief agitation by vortexing, after 240 s (4 min) and incubation for a further 26 min; c: Sensitivity of the dipstick recombinase polymerase amplification (RPA) assay for NSD phytoplasma. The RPA assay was assessed against a dilution series of the NSD phytoplasma from 10,000 to 1 copies of the *imp* gene. Reactions were incubated at 39 °C for 1800 s (30 min) and amplification visualized on lateral flow strips (dipstick) with a biotin capture.

(data not shown).

3.3.5. Comparative analysis of real-time PCR, nested PCR and RPA

The diagnostic performance of the nested 16S rDNA PCR assays for NSD was evaluated against the *imp*-based real-time RPA assays developed in this study. Sixty-six Napier grass samples (Table S1) from various geographical locations in Western Kenya were analyzed for NSD infection using the three above-mentioned diagnostic assays. Nested PCR was regarded as the reference (Gold standard) test and the other diagnostic protocols were compared to it. According to the results displayed in Table 2, nested PCR and real-time PCR had comparable detection efficiencies (89.4%). Among the 7 samples, which tested negative by nested PCR, 3 of them tested positive by real-time PCR and 4 by RPA. Real-time PCR showed a sensitivity of 94.9% and specificity of 57.1%, with PPV of 94.9%, and NPV of 57.1%. The real-time RPA method had a sensitivity and specificity of 100% and 57.1% respectively, with PPV of 93.6% and NPV of 100%.

3.4. Serological testing

3.4.1. Expression and purification of recombinant IMP

From the his-tagged full-length *imp* gene in pQE30 a protein of 17.6 kDa was expressed in *E. coli* Bl21λDE3 using standard parameters. Most of the protein was produced as insoluble inclusion bodies but under denaturing conditions using Ni-NTA affinity chromatography, more than 3 mg of IMP were recovered from a 300 ml bacterial culture. The removal of urea by dialysis caused partial precipitation of IMP, but this did not affect immunization and screening procedures.

3.4.2. Production of IMP-specific monoclonal antibodies

The supernatant of 26 hybridoma clones showed ELISA readings of more than 2 OD 30 min after substrate addition. IgGs from all supernatants were tested against native IMP extracted from Napier grass infected with NSD strain Mbita1 and healthy control plants. The IgGs of all 26 clones reacted with a protein of about 17 kDa in immunoblot assays with NSD-infected Napier grass but not with proteins from healthy Napier grass. Two clones, hereafter referred to as mAb28.2 and mAb37.2, showed the strongest reactions and were selected for preparative monoclonal antibody production. Two milligrams of each mAbs were produced and the antibody concentration was adjusted to 1.4 μg/μl and 1.2 μg/μl for mAb28.2 and mAb37.2, respectively.

3.4.3. Specificity of selected mAb 28.2 and 37.2 and their cross-reaction to other phytoplasma species tested by immunoblot assays

To demonstrate the performance of the mAb 28.2 and mAb 37.2 for IMP detection in Napier grass field samples SDS protein extracts of 10 phytoplasma-infected Napier grass accessions and two healthy Napier grass controls were examined (Table S1). The protein concentration of SDS. extracts was 10 ng/μl and 50 ng of total protein was separated by PAGE. All NSD-infected field samples, the reference strain Mbita1 and the recombinant IMP showed a distinct reaction with the mAb28.2 and mAb37.2 (Fig. 5). Both antibodies reacted with the same signal intensity to the antigen of infected samples. In some of the lanes (Fig. 5, lanes 5–8, 13 and 14) an additional band with a molecular weight of about 37 kDa was apparent representing most likely dimers of IMP. The recombinant IMP had a 0.8 kDa higher molecular weight due to the histidine tag (Fig. 5 lane 1).

The specificity of the mAbs 28.2 and 37.2 to the target protein was assessed in cross-reactivity tests with phytoplasma-infected periwinkle accessions by western blot analysis. The presence and quantity of phytoplasmas in the accessions had been verified by real time PCR (10^{10} - 10^{11} phytoplasmas/g plant tissue). The protein concentration of SDS extracts was about 6 ng/μl and 50 ng of total protein from periwinkle and Napier grass plant accessions were separated. Both antibodies reacted only with Napier grass infected with NSD strain Mbita1 and the recombinantly expressed IMP. None of the periwinkle samples showed a positive reaction (data not shown).

To determine the limiting amount of monoclonal antibody necessary to create a positive reaction in western blot experiments, mAb28.2 and mAb37.2 were diluted 1:1,000, 1:5,000, 1:10,000 and 1:50,000 in blocking solution representing 1.4 μg, 280 pg, 140 pg and 28 pg for mAb28.2 and 1.2 μg, 240 pg, 120 pg and 24 pg for mAb37.2 per ml blocking solution, respectively. The immunoblots carrying 50 ng and 5 ng of SDS protein extracts from Napier grass infected with NSD strain Mbita1 were incubated with 2.5 ml of antibody containing blocking solution. . Both antibodies gave positive results in all dilutions with 50 ng and 5 ng of plant proteins, although the intensity of the 1:50,000 dilution was compellingly weaker than with higher concentrations (Fig. 7).

3.4.4. Dot blot analysis and ELISA

To examine the suitability of the mAb28.2 and mAb37.2 for dot blot and ELISA analysis PBS extracted non-denatured protein samples were tested. Undiluted and diluted protein extracts of sample NSD strain Mbita1 (Table S1) and healthy Napier grass were spotted on nitrocellulose membranes and incubated with both monoclonal antibodies diluted 1:5,000, 1:10,000 and 1:50,000. Both antibodies reacted distinctly with all dilutions to undiluted and 1:10 diluted samples of the NSD strain Mbita 1. A faint reaction was visible when the NSD-positive extract was diluted 1:100 with the antibody dilutions 1:5000 and 1:10,000 (Fig. 6). The healthy control did not react with the antibodies but in the undiluted sample a staining was visible due to the strong green color of the plant protein extract.

A direct ELISA assay was performed with the same sample

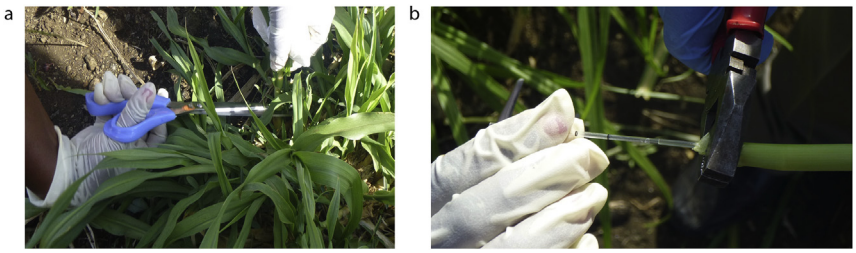

Fig. 3. a: Sampling of Napier grass in the field for detection of NSD phytoplasma. Stem cuttings of Napier grass stems were obtained using a pair of scissors sterilized with 10% bleach. b: Aseptic collection of Napier grass phloem sap samples in a field set-up for detection of NSD phytoplasma. A pair of pliers sterilized with 10% bleach was used to squeeze the stem cuttings and the sap immediately sucked-up into a sterile capillary tube.

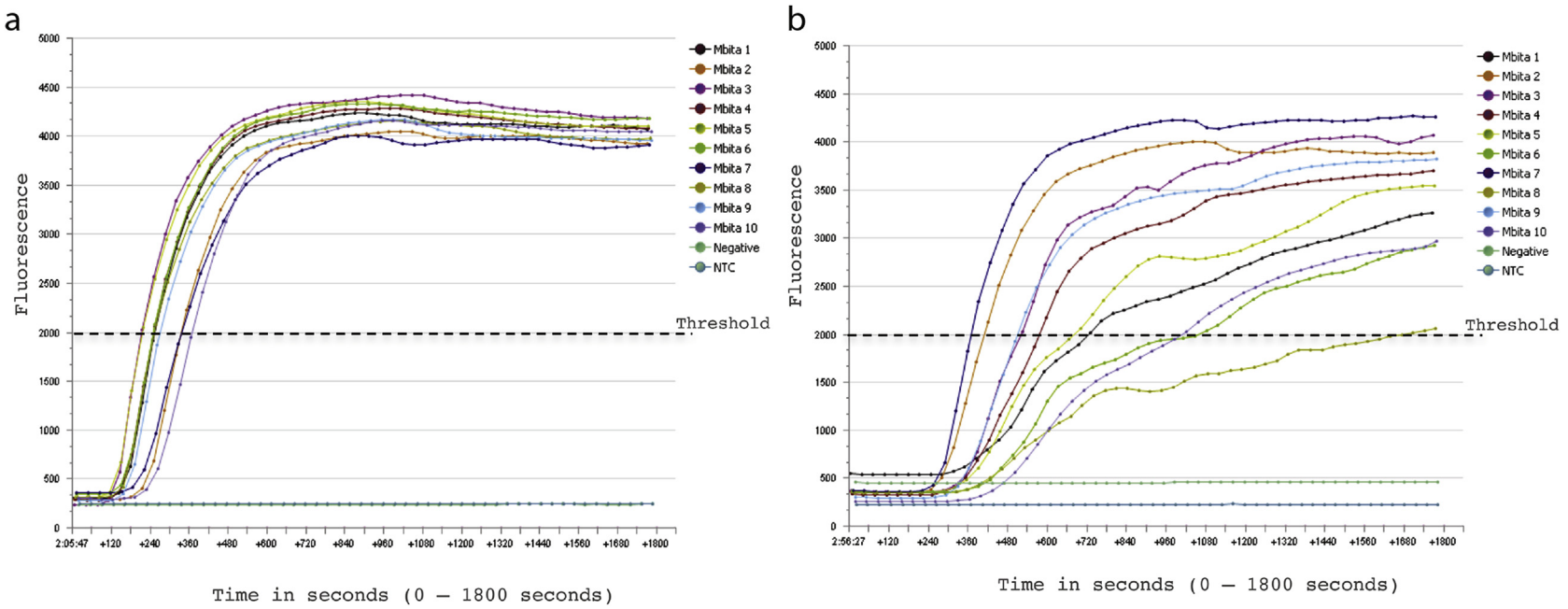

Fig. 4. RPA assay for NSD phytoplasma detection in 10 plant samples infected with NSD phytoplasma (Mbita 1–10), one healthy plant and a no template control (NTC). a: amplification of NSD phytoplasma using 1 μl of DNA extracted by the CTAB method, b: amplification using 1 μl of phloem sap.

Table 2
Comparative analysis of efficacy of three diagnostic methods for detection of Napier grass stunt disease (NSD). The table depicts results of 66 Napier grass DNA samples analyzed for NSD infection by real-time PCR and recombinase polymerase amplification (RPA), relative to nested-PCR as the reference assay. Sensitivity, specificity, positive and negative predictive values are shown [36]. TP; True positive, FP; False positive, FN; False negative, TN; True negative, PPN; Positive predictive value and NPV; Negative Predictive value, CI = 95%.

Diagnostic assay	Values in percentage (%) (95% CI)						
	Nested PCR			Sensitivity	Specificity	PPV	NPV
	Results	Positive	Negative				
Real time PCR	Positive	56 TP	3FP	94.9	57.1	94.9	57.1
	Negative	3 FN	4 TN				
Real time RPA	Positive	59 TP	4FP	100	57.1	93.6	100
	Negative	0 FN	3 TN				

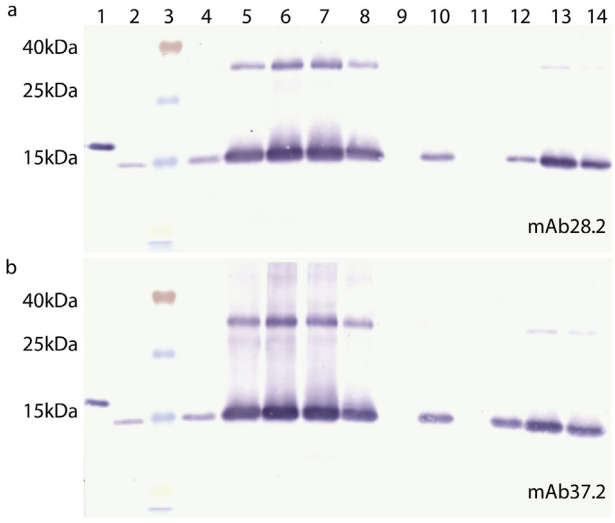

Fig. 5. Western blot analysis of 10 NSD-infected accessions with monoclonal antibodies mAb28.2 (A) and mAb37.2 (B) diluted 1:2500 in blocking solution. Samples from left to right: 1, recombinant IMP; 2, NGS1-JKI; 3, prestained protein marker; 4–8, Samples obtained from icipe; 9, 11, healthy Napier grass; 10, 12–14, extracts K-6 to K-10.

extracts as used for dot blot experiments (Fig. 6). Although, the plant extracts were diluted with coating buffer instead of PBS buffer. Extracts from healthy and phytoplasma-infected plants were serially diluted up to 1:100,000. The mAb dilutions were kept constant at a dilution of 1:5000. Readings significantly above the healthy control value ($E_{405} = 0.09$, $n = 4$) were obtained for both antibodies up to a sample dilution of 1:100 ($E_{405} = 0.35$, $n = 2$). The absorbance in the 1:1000 dilution dropped to 0.09 to 0.1 similar to the healthy control.

4. Discussion

Phytoplasmas are wall-less bacteria affecting several hundreds of plant species worldwide, many of agricultural and economic importance. Specific, sensitive and rapid diagnosis is therefore of utmost importance to control and hinder the spread of the disease. Phytoplasmas cannot be cultured at the moment in axenic media which make these organisms difficult to work with. Consequently, diagnostic tests rely on the detection of phytoplasma specific molecules such as nucleic acids or proteins. Molecular and serological methods such as conventional PCR, real-time PCR and ELISA tests have been developed and used for the detection of phytoplasma-induced diseases [10,29,38,39]. Nevertheless, the use of these tests in low and middle-income countries is greatly limited by the need for laboratory infrastructure, high costs and well-trained staff.

In Eastern Africa, detection of NSD is performed in only a handful of laboratories using nested PCR and real-time PCR assays based on the 16S ribosomal gene with reasonable sensitivity. Recently, a LAMP-PCR assay was described [19], but its specificity was low, and no data on sensitivity were provided. In the absence of a field-applicable and easy to use reliable diagnostic tests, NSD phytoplasma will spread rapidly in the fields and can only be detected at advanced stages, usually after the first cutting of infected Napier grass when the regrowth begins to exhibit the characteristic symptoms of stunting, yellowing, proliferation of tillers and shortening of internodes [2].

Non-ribosomal nuclear genes remain under-exploited as alternative markers for molecular (i.e. PCR-based) detection of phytoplasmas. According to a previous report by Kakizawa and coworkers [40], nuclear genes in phytoplasma genomes are predominated by genes encoding highly-antigenic surface exposed membrane proteins, referred to as immunodominant membrane proteins. Apart from being abundant, immunodominant membrane protein genes are also highly expressed during phytoplasma infection [41]. They are likely to interact with host proteins and determine the transmissibility of the pathogen [42]. Immunodominant membrane proteins are encoded by three distinct genes i.e. *imp*, *idpA* and *amp*. As each phytoplasma harbors a unique set of immunodominant membrane proteins they present candidate diagnostic targets for inclusion into diagnostic assays.

In this study, we developed a field-applicable molecular assay targeting the *imp* gene to provide the means for the diagnosis of the Napier grass stunt phytoplasma, a strain of *Candidatus* Phytoplasma oryzae, in affected regions in East Africa. One of the priorities was to simplify the sample preparation step that currently requires elaborate DNA extraction steps, because most plants are known to be rich in polysaccharides, phenolic compounds and other substances that inhibit PCR reactions [43]. This necessitates the use of either expensive commercial kits, or hazardous chemicals like CTAB, phenol and chloroform, which cannot be achieved on-farm or in basic laboratories without specialized safety cabinets. We explored a simple sample preparation procedure that employs tools available at every farmers' household, such as a knife, a pair of scissors,a pair of pliers and household bleach. The above implements were used to extract phloem sap, from the grass stems easily and samples were aseptically collected in less than 1 min. The sap was used directly in the RPA reaction in place of DNA, reducing the total assay time to a maximum of 30 min. This approach not only accelerated the time taken for diagnosis, but also reduced the overall assay cost, and increased the safety of the assay by omitting the use of hazardous chemicals. This procedure was also very attractive compared to current PCR-based diagnostic assays that require more handling steps necessitating skilled molecular biologists, specialized equipment and infrastructure.

Our results demonstrate, for the first time the direct isothermal

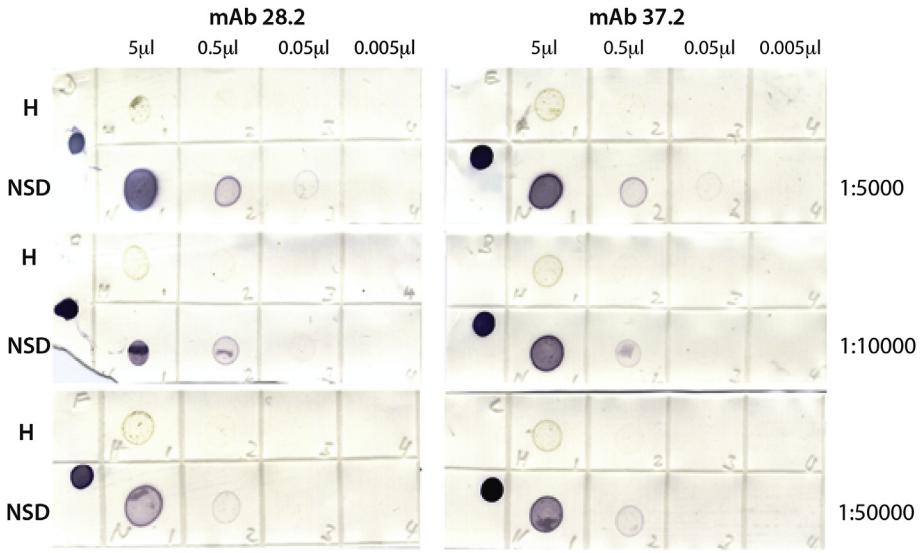

Fig. 6. Dot blot experiments of diluted PBS-extracted protein samples NGS3-JKI (NGS) and Kh-1 (H) with mAb28.2 and mAb37.2 dilutions indicated on the left. The dark dot on the left side of the blots represents recombinantly expressed IMP. Fife microliter of the undiluted sample contained 50 ng of protein. The dilutions thereof contained 5 ng, 0.5 ng and 0.05 ng, respectively.

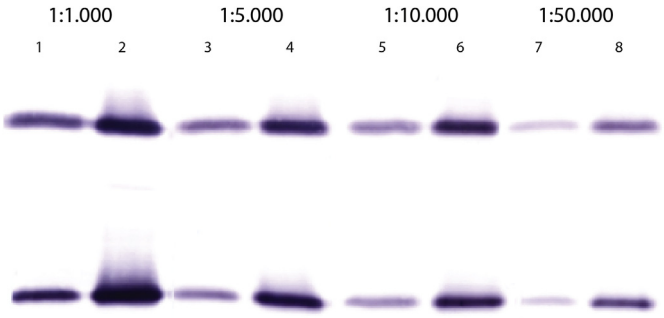

Fig. 7. Dilution series of monoclonal antibodies mAb28.2 (top) and mAb37.2 (bottom) tested against 50 ng and 5 ng of SDS protein extract from NSD sample K-9. Lanes 2, 4, 6 and 8 contained 50 ng of total SDS-proteins. Lanes 1, 3, 5 and 7 contained 5 ng of protein.

detection of phytoplasmas in plant sap, providing a means for monitoring of phytoplasma and other phloem-limited pathogens in large scale. The potential of pathogen detection in plant sap through microscopy and PCR assays, was earlier demonstrated for bacterial and viral infections [44] as well as xylem-limited bacterial pathogens [45], and recently, other unculturable pathogens [46]. Compounds in the phloem sap seem not to inhibit the recombinase polymerase amplification (RPA) assay and RPA has been applied successfully on sap and crude plant extracts to detect viral [47,48], bacterial and fungal pathogens [49,50]. Our method is a valuable detection method, because of its ease of use in the field and its rapidity compared to regular PCR assays. However, the technique is not inexpensive.

We demonstrated the analytical specificity and sensitivity of the RPA assay developed for NSD diagnosis. The specificity of the assay was assessed using samples from other phytoplasma species, among them the closest relatives to NSD phytoplasma, the rice yellow dwarf phytoplasma and Bermuda grass white leaf phytoplasma. Except NSD accessions the DNA of no other *Ca*. Phytoplasma species was amplified indicating the RPA assay specificity. Our assay detected as little as 10 copies of the gene per 100 ng of total extracted DNA and is similar sensitive to detection assays of three plant pathogens [50].

Regarding diagnostic accuracy, RPA had the highest diagnostic sensitivity (at 100%) and was more sensitive than the 16S-based real-time PCR. This implies that the RPA assay can reliably be used to detect all true positives to the same level as the reference nested PCR assay. The high diagnostic sensitivity of the RPA assay also indicates that a negative result can be reliably interpreted as absence of NSD infection with 100% accuracy. However, the diagnostic specificity was lower (at 57.1%) indicating that the test was likely to detect more positives and misses out some true negatives in comparison to the reference nested PCR test. This may also imply that RPA can detect weak positives that may be missed out by other tests, which would be desirable in the detection of early stage disease in a farm or nursery.

We successfully demonstrated high yield of phytoplasmas in the sap and the feasibility of using crude sap directly extracted from infected plants for detection. The test was positive for all samples, whether DNA or crude sap extracts were used as a template. The only difference was the detection time, which was delayed when sap was used, but which is an acceptable trade off given the indispensability of laborious DNA preparation. Our results demonstrate that detectable signals were seen within 12 min in infected samples whether DNA or sap was used, implying that an assay time of 12–20 min, instead of the routine 30 min used in RPA assays, was adequate to diagnose NSD. For easy read out of the results, we combined the RPA assay with a lateral flow device. This led to a slight decrease in sensitivity, with the lateral flow device requiring between 10 and 100 copies of target gene to show a positive line. Nevertheless, due to the number of phytoplasmas in the plant material, this is unlikely to impair the detection.

Finally, our test has been demonstrated to work on crude leafhopper extracts too, which makes it a valuable tool for pathogen vector research. Monitoring of phytoplasma-positive leafhoppers is necessary to record their distribution especially in the light of global warming and changes in vector habitats. Nevertheless, the taxonomic identification of leafhopper species is difficult and research staff instead of farmers should identify phytoplasma-positive leafhoppers. The concentration of phytoplasmas in leafhoppers seems to be lower than in Napier grass and interestingly not all leafhoppers that fed on infected plants gave positive results

in the detection of *Ca.* P. oryzae. This should be followed up by subsequent trials, investigating this in more detail. Indeed, the leafhoppers that do not contain *Ca.* P. oryzae could be tested to investigate new strategies for fighting the disease.

We also established a serological laboratory assay for the detection of the NSD phytoplasma. Serological methods, especially ELISA, have been applied for the detection of phytoplasmas since the early 1980's [51]. The versatility and common knowledge on the use of immunological detection procedures in plant pathology prompted us to develop monoclonal antibodies against the IMP protein of NSD. Polyclonal antisera and monoclonal antibodies have been successfully used for the detection and differentiation of several phytoplasma diseases, such as peach yellow leaf roll, clover phyllody [51], rice yellow dwarf phytoplasma [52], apple proliferation phytoplasma [29], and cape St Paul wilt disease phytoplasma [53] to name a few. In this study, two specific monoclonal antibodies designed to detect the IMP protein in infected Napier grass were produced. The choice of IMP as the target protein was based on the high sequence conservation of this gene within NSD accessions and the distinct difference to other phytoplasma *imp* gene sequences. The monoclonal antibodies mAb28.2 and mAb37.2 proved to be specific for the NSD pathogen and showed a high affinity to the target protein. The detection of native NSD IMP with picogram amounts of antibodies in microgram quantities of plant tissue extract demonstrates the power of the antibodies and the abundance of the protein. It is intended to incorporate the monoclonal antibodies into a lateral flow detection assay like for the RPA assay developed during this study, so that both systems can be applied interchangeably under field conditions according to the user's preference.

5. Conclusion

This is the first report of an RPA based assay for the detection of the Napier grass stunt phytoplasma. The assay is based on the amplification of an *imp* gene fragment, highly specific for this pathogen. Moreover, we demonstrated that this assay can be used on phloem sap directly squeezed from infected plant material and that the pathogen, can be detected by RPA in real time mode or by a lateral flow device. The latter makes it a very valuable tool for fast and easy on-farm detection. We also developed a specific and sensitive serological test with monoclonal antibodies specific to the IMP protein. Like RPA this test has the potential to be developed for a dipstick assay.

Novel diagnostic tests are crucial for improved disease control [44] and the easier and more robust diagnostic tests become the better is the acceptance by the research community and its implementation in official diagnostic regulations.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mcp.2017.06.004.

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