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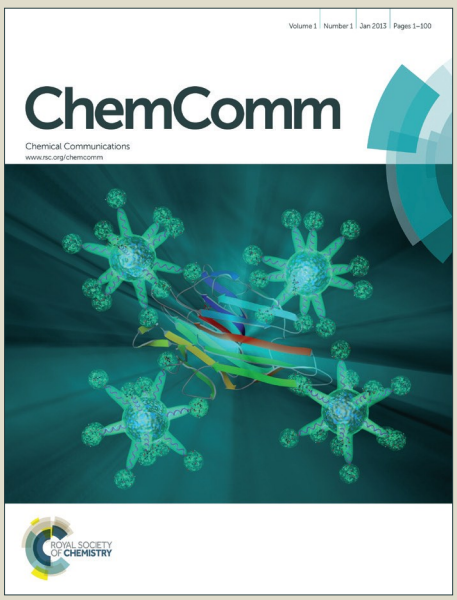

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Re-purposing bridging flocculation for on-site, rapid, qualitative DNA detection in resource-poor settings

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E. J. H. Wee,[a,]† H. Y. Lau,[a,b,]† J. Botella[b] and M. Trau[a,c]

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Developing molecular diagnostics in resource-poor settings is challenging. As such, we purpose-built a novel bridging flocculation assay for qualitative evaluation of isothermally amplified DNA by naked eye. The flocculation assay was dependent on pH, DNA polymer amounts and lengths. The method was first applied to the rapid and sensitive detection of important plant pathogens and subsequently extended to other pathogens across the animal kingdom to demonstrate the wide applications of our approach.

Nucleic acid point-of-care bioassays that can be performed on-site are in high demand.[1] However the challenge is further confounded in resource-poor settings due to the lack of infrastructure and skilled labor. To address this, new methods tailored for low resource settings requires considerations from sampling to detection/amplification and to evaluation of results. The agriculture industry is one area in urgent need of bioassays requiring minimal infrastructure especially in agriculturally reliant developing regions.[2] Traditionally, an experienced plant pathologist identifies the disease by a subjective visual examination of disease symptoms.[3] To address this, more analytical diagnostic methods have since been developed.[4-10] However, these methods require expensive and sophisticated equipment and can only be performed in specialized laboratories by well-trained technicians. This results in delayed intervention which may eventually lead to the loss of the entire crop harvest.

As the distinction between diseased and healthy samples is binary, a readout method mirroring a digital yes/no result may be useful. Herein we describe a novel method to cheaply visualize amplified disease-specific DNA/RNA with minimal equipment via bridging flocculation. A key characteristic of flocculation is the abrupt transition from solution phase to flocculate which makes this phenomena ideal for binary yes/no applications. To the best of our knowledge, the detection of DNA/RNA has not yet been demonstrated via a DNA-mediated bridging flocculation mechanism which can be readily observed by the naked eye (Fig 1a). Indeed, it is the unique feature of the bridging flocculation process to discriminate between long and short DNA polymer segments which lies at the heart of enabling a very attractive, versatile, field-ready system for the detection of any pathogen DNA or RNA sequence.

Bridging flocculation is a well-known phenomenon in colloid chemistry and is used for a wide variety of colloidal separation processes (e.g., to clarify contaminated water). The phenomenon was first described in the 1950's by Ruehrwein, R.A[11] and explained in the 1960's by La Mer and Healy[12-14] to be the result of the surface adsorption of polymers which are long enough to cross-link multiple particles together and thus (reversibly) flocculate out of solution. A key aspect of this phenomenon is that the polymers (in our case DNA amplicons) must be of sufficient length to induce this flocculation. DNA primer pairs, by contrast, are typically too short to enable this type of particle cross-linking, hence flocculation can only occur if the primer pairs are successfully amplified to create long polymer strands, which in turn, represents the presence of an offending DNA sequence. Another key aspect of bridging flocculation is that the solution conditions (e.g., salt concentration and pH) must be adjusted so that polymer/surface interactions are stronger than the polymer/solution interaction (as defined by the relevant Flory-Huggins Parameters[15-17]). Under, such conditions longer polymer chains (DNA amplicons) will displace surface adsorbed shorter polymers (primers) to induce a spontaneous flocculation. As a consequence, the bridging flocculation process is also reversible and may have the added versatility to "tune" the assay possibly for quantitative applications.

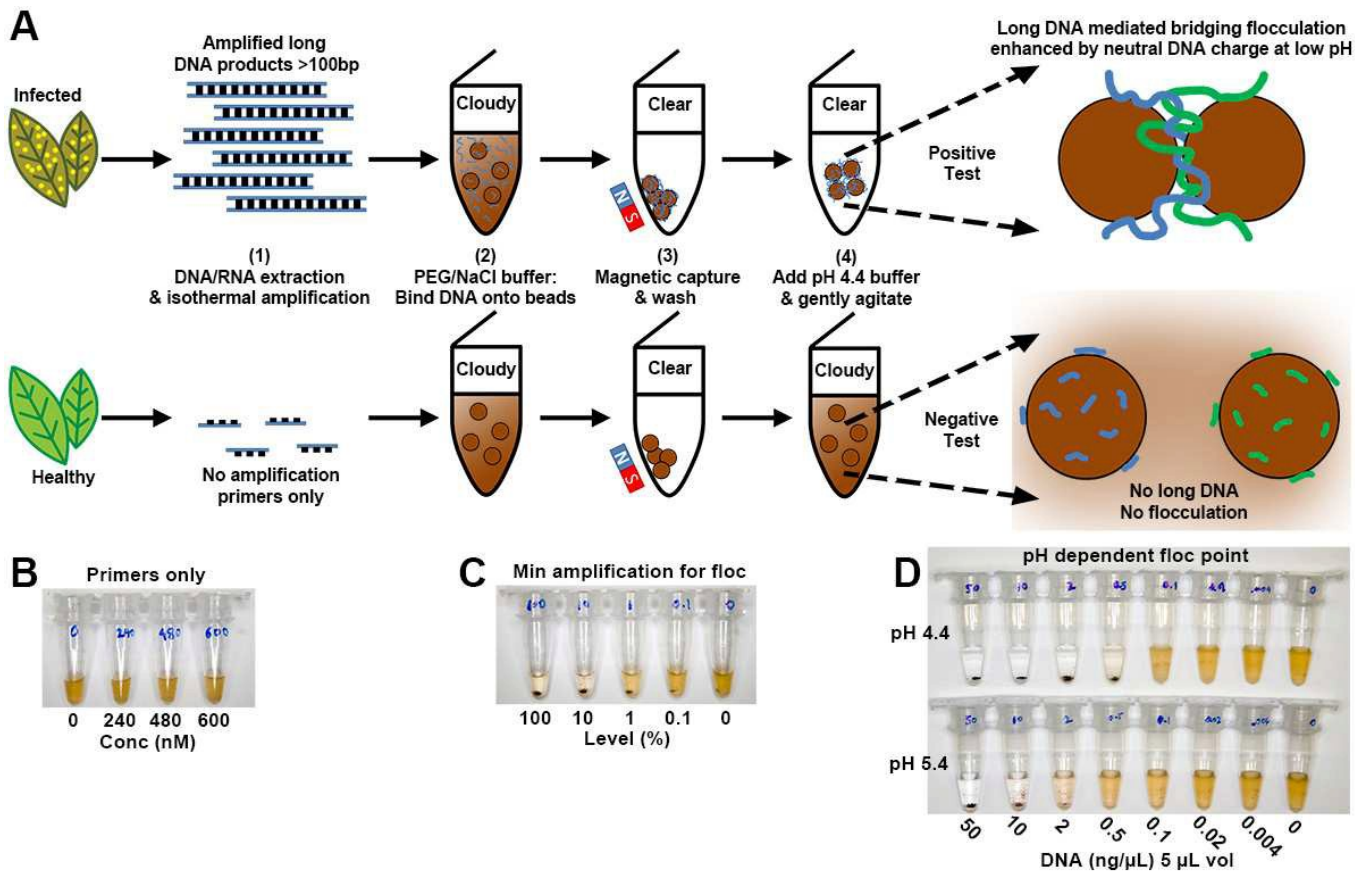

Fig 1. The bridging flocculation assay. (A) Conceptual representation of the DNA-mediated bridging flocculation assay which only occurs in the presence of pathogen DNA which is subsequently amplified to produce high molecular weight DNA amplicons/polymers. (B) Excess primers (low molecular DNA polymers which have not been amplified) do not induce flocculation. (C) Only 10% or higher amplification results in flocculation. (D) Cut-off concentration of amplified DNA for DNA-mediated bridging flocculation is pH-dependent. Each figure is a representative of at least 3 experimental replicates.

As a proof-of-concept we used the Solid Phase Reversible Immobilization (SPRI)[18] method of DNA purification to first select for DNA lengths above 100 bp. Briefly (see ESI for detailed method), high molecular weight DNA were first precipitated onto the bead surface in a high polyethylene glycol (PEG)/NaCl buffer. DNA loaded beads were next enrich with a magnet and the PEG/NaCl was removed with an ethanol wash. Then instead of eluting the captured DNA, we used a low pH acetate buffer to trigger DNA loaded magnetic beads to flocculate while long amplicon-free beads are readily dispersed back into solution. To confirm that only amplified amplicons of lengths longer than primers could trigger flocculation, we tested the assay using only primers (Fig 1b). As expected, even up to 600 nM of primers were inert to the flocculation assay. Next, to determine how much amplification was required to trigger the flocculation, various ratios of product to primers mixes representing various levels of amplification were evaluated. As little as 10% amplification efficiency for a 250 bp amplicon was enough to trigger a visually distinct positive response (Fig 1C). This was estimated by assuming that the maximum amount of amplified products was equivalent to the initial primer amount (480 nM) in the reaction. We also observed that long single stranded DNA such that produced by rolling circle amplification, could also mediated a flocculation response (ESI Fig S2). Therefore, this flocculation assay could potentially be a universal readout of high molecular weight DNA produced by a plethora of amplification systems.

Another interesting feature of the assay was its sensitivity to pH changes (Fig 1D). At pH 4.4, 0.5 ng/µL (5 µL volume) of amplified product could be detected. However, at pH 5.4, the cut-off concentration for clear and distinct flocculation increased 20-fold to 10 ng/µL (5 µL volume). In addition, titrating pH with NaOH reversed flocculation (results not shown). Thus, this feature could offer some level of "tuning" and may be beneficial for certain applications. This pH versatility is however, absent in current nanoparticle approaches. Considering these observations: (1) the requirement for long DNA polymers, (2) pH dependence, (3) reversible agglutination and (4) sharp transition between solution phase and flocculate, we concluded that the mechanism was a DNA-mediated bridging flocculation of the particles.[19, 20] We hypothesized that the accumulation of beads to the magnet facilitates the DNA/bead entanglement by bringing neighbouring DNA/beads into close proximity such that DNA strands from one bead may also facilitate interaction with adjacent beads. On introduction of an acidic buffer eg. acetate buffer ph4.4, two mechanisms may occur to enhance flocculation: (1) DNA charge is neutralized at ph4.4, thus making the bead/DNA surface hydrophobic and will spontaneously flocculate in an aqueous environment. (2) Precipitated high molecular weight DNA "intertwine" on the beads surface to form an aggregate or flocculate. However, DNA-free beads which have the COOH groups exposed, remain negatively charged thus electrostatically repel each other and readily dispersing into solution.

Besides the mechanism of aggregation, bridging flocculation is also distinct in many ways from many recently described aggregation

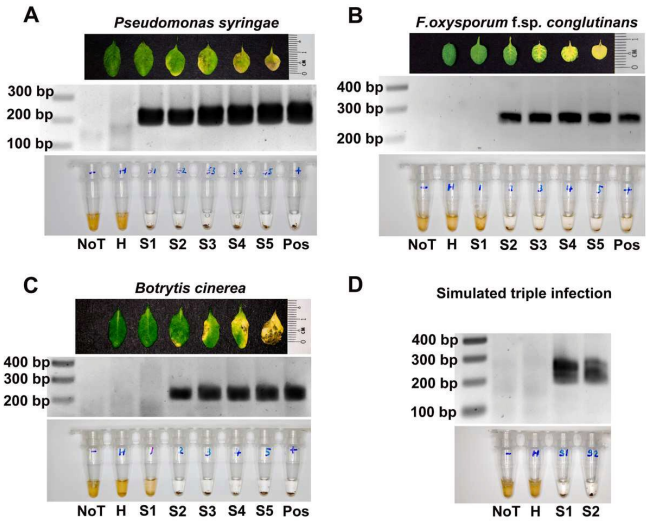

Fig 2. Performance at detecting three plant pathogens in *Arabidopsis thaliana*. (A) *Pseudomonas syringae*, (B) *F.oxysporum* f.sp. *conglutinans,* (C) *Botrytis cinerea*, (D) simulated triple infection. Top row: photographs of leaves at various times after infection, S1 to S5. H: healthy sample. Pos: positive control. NoT: no template control. Middle row: gel electrophoresis images of corresponding RPA reactions performed on the same leaf. Bottom row: photographs of the flocculation assay corresponding to the RPA reactions. Each figure is representative of at least 3

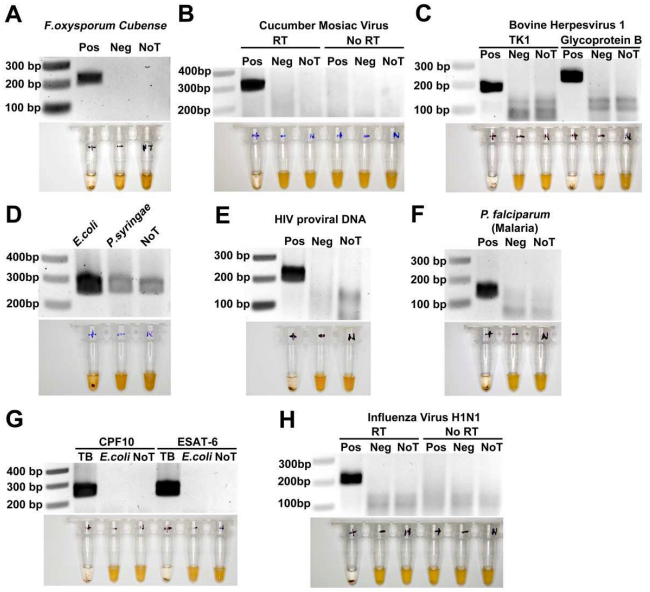

Fig 3. Detecting multiple disease pathogens from across various host kingdoms. (A) *F. oxysporum cubense* Race 1 in banana stems. (B) *Cucumber mosaic virus* in *Nicotiana benthamiana* leaves. (C) *Bovine Herpesvirus 1* in bovine cells using two pathogen target genes, tyrosine kinase 1 (*TK1*) and Glycoprotein B. (D) *E. coli* in water using *P. syringae* as an unrelated control. (E) *HIV* proviral DNA in Jurkat cells. (F) *P. Falciparum* blood cultures. (G) *Mycobacteria Tuberculosis* in cultures using two pathogen target genes, CFP10 and ESAT-6. *E. coli* was used as an unrelated control. (H) Influenza virus H1N1 in culture media. Top row: gel electrophoresis images of RPA reactions performed. Bottom row: photographs of the flocculation assay corresponding to the RPA reactions. Pos: positive sample. Neg: negative sample. NoT: no template control. RT: reverse transcriptase. Each figure is representative of at least 3 experimental replicates.

assays for biomolecule detection using gold nanoparticles via various strategies including antibody, DNA probe modified- and electrostatic-mediated aggregation.[21-24] For instance, unlike gold nanoparticle methods, the larger size and variety of colloidal particles/material which can be manipulated by a bridging flocculation process (e.g., the 1 µm sized particles used here) allows for better naked eye contrast and therefore does not require the use of spectrometry equipment to verify flocculation hence making the bridging flocculation assay ideal for resource-poor applications.

To enable a meaningful application, we married the robust isothermal recombinase polymerase amplification (RPA)[25], as a proof of concept, with our flocculation assay to detect some examples of agriculturally important pathogens. The RPA was used to facilitate the generation of large amounts of long DNA polymers (amplicons) that could trigger a flocculate only if the pathogen was present. To this end, we first analysed the model plant *Arabidopsis thaliana* infected with different pathogens at various degrees of infection severity. This was achieved by collecting leaf samples at various time points post infection (S1-S5, see ESI for details)[26]. This approach also served both as a typical traditional visual diagnosis method, and to emulate situations when a farmer would want a diagnosis performed.

To enable a sampling procedure with minimal equipment, we used a modified SPRI approach to extract total DNA from leaf cuttings (see ESI for detailed method description). This was then followed by the isothermal RPA amplification of pathogen-specific sequences at 37°C for 15 minutes. With this approach we could detect the bacterial pathogen *Pseudomonas syringae* very early in the infection process, even before disease symptoms manifested (Fig 2A). To verify that a flocculation was indeed a result of successful RPA amplification, an aliquot of the RPA reactions was also visualized via gel electrophoresis. As expected, flocculation occurred only when there was a successful RPA amplification, therefore confirming that the flocculation assay could be used a viable proxy to evaluate successful amplification which in turn, indicates the presence of the offending pathogen. Compared with current methods in the literature for detecting *Pseudomonas syringae* in plants, our approach is the fastest with comparable, if not better, sensitivity as other previously described methods (ESI Table S2).

In addition, the presence of two other important and devastating pathogenic fungi, *Fusarium oxysporum f. sp. conglutinans* and *Botrytis cinerea* were also detected very early in the infection process when symptoms were just visible to the human eye (Fig 2B & Fig 2C). In contrast, an additional validation by qPCR could only detect Fusarium infection at a later time point using the same amount of starting material and primers (ESI Fig S3). While we could not reproducibly detect the earliest (S1) time point for *F. oxysporum* and *B. cinerea* (Fig 2B and C), we do not view it as a major limitation since plants at these early disease stage were virtually symptomless, hence would have gone unnoticed by the farmer. In actual farming situations, disease diagnostic assays are only performed when potential disease symptoms appear.

To demonstrate a triplex detection assay, leaves from three plants inoculated with different pathogens were pooled together at their respective times post infection to simulate a triple infection. We could reliably detect the presence of pathogens in plants with early signs of infection (Fig 2D, ESI Fig S4). These results demonstrate both the feasibility and sensitivity of the assay in early detection of plant infections i.e. when phenotypic symptoms were just beginning to manifest. While the assay performed well on the *Arabidopsis thaliana* plant model system, we wondered if the assay could be applicable to commercial crops and non-leaf tissues with different composition and putative assay inhibitors. To this end, we tested the approach on Lady Finger banana stem cuttings from diseased field plant samples to detect *F. oxysporum f. sp. cubense* Race 1. We were able to distinguish healthy from diseased sample, thus supporting the

potential for a viable on-site field test for agricultural applications (Fig 3A). Current practices for crop pathogen identification are done via ELISA or PCR-based methods performed off-site at central facilities.[2, 4-10] While useful, the time delay (days) to obtaining results is not ideal as timely interventions are crucial for preventing catastrophic crop losses.[2] In contrast, our approach has the potential for on-site applications because it uses an isothermal amplification method coupled with a simple naked eye evaluation method that can be performed with minimal equipment in under 90 minutes. Recently, RPA has been performed using only body heat.[27] This coupled to a low resource DNA mediated bridging flocculation evaluation assay such as the one described herein could further advance low cost on-site molecular diagnostics.

As a large number of important pathogens use RNA as their genetic material we turned our attention to RNA-based pathogens. By adding MMLV reverse transcriptase (RT) to the RPA mix we performed RT-RPA[28, 29] on *Nicotiana benthamiana* plants infected with cucumber mosaic virus, a RNA virus that affects a multitude of commercial vegetable crops. As was the case with DNA-based pathogens, only samples with viral infection but not healthy plants tested positive (Fig 3B). Finally we extended the approach to the detection of a wider variety of targets. These included bovine herpes virus 1 in bovine cells (Fig 3C), *E.coli* in water (Fig 3D); and of human diseases such as proviral HIV (Fig 3E), malaria (Fig 3F), *Mycobacteria tuberculosis* (Fig 3G) and influenza virus H1N1 (Fig 3F), all of which we could successfully discriminate between infected and uninfected samples using the flocculation assay to visualize positive amplifications.

Conclusions

In conclusion, we have described a novel bridging flocculation assay for naked eye qualitative evaluation of amplified DNA. The combination of RPA with the flocculation assay then forms the basis of a simple strategy for on-site nucleic acid diagnostics with minimal equipment that may find wide applications. This strategy was first applied successfully to detect economically important plant pathogens and then extended to detect a suite of pathogens from a variety of sources. While promising, a current limitation of the method is need for multiple wash steps which can be resolved in future improvements to the method. However, considering the wide range of pathogens and samples demonstrated here, we believe the assay has the potential for on-site, low resource applications.

Notes and references

[a]Centre for Personalized Nanomedicine, Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, QLD 4072, Australia.

[b] Plant Genetic Engineering Laboratory, School of Agriculture and Food Sciences, The University of Queensland, QLD 4072, Australia.

[c] School of Chemistry and Molecular Biosciences, The University of Queensland, QLD 4072, Australia.

† Authors contributed equally.

Emails: m.trau@uq.edu.au, j.botella@uq.edu.au

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