Recombinase polymerase amplification technology: Assessment for nucleic acid-based point-of-care diagnostics

Thèse

Rana Daher

Doctorat en microbiologie-immunologie
Philosophiae Doctor (Ph.D.)

Québec, Canada

Résumé

Cette thèse de doctorat porte dans l'ensemble une étude approfondie sur une technologie émergente pour l'amplification isotherme des acides nucléiques appelée *recombinase polymerase amplification* (RPA). L'introduction porte une description détaillée sur la RPA. Cette revue de littérature documente et discute les diverses applications de la RPA en soulignant les connaissances actuelles concernant les applications diagnostiques. Malgré la composition complexe de la RPA (6 à 7 protéines dans le même mélange réactionnel), cette dernière s'avère une technologie rapide (générant des résultats <20 min), spécifique et sensible (détection de l'ordre de quelques copies de génome), et largement appliquée dans différentes disciplines. Ces avantages nous permettent de croire que la RPA possède la flexibilité nécessaire pour être utilisée comme outil de diagnostic rapide des maladies infectieuses en réduisant le temps d'obtention des résultats à moins d'une heure au lieu de 2 à 3 jours avec les tests de cultures standards. En conséquence, il sera possible d'intégrer la RPA dans des plateformes microfluidiques ou laboratoire sur puce qui permettent la préparation d'échantillons, l'amplification et la détection des acides nucléiques des microbes causant des infections. En premier lieu, les travaux de cette thèse ont généré des lignes directrices additionnelles pour la conception des amorces/sondes RPA. En second lieu, nos travaux ont permis de développer un essai diagnostic RPA pour la détection des streptocoques du groupe B, responsables de la septicémie et la méningite chez les nouveau-nés. Cet essai fut le premier à évaluer la performance de la RPA avec des échantillons cliniques humains. Ce test diagnostic RPA a été comparé à une méthode de référence, la réaction en chaîne par polymérase (PCR). Cette démonstration sur des échantillons cliniques nous à inciter à pousser notre étude pour réaliser le dernier objectif de ce projet qui consistait à automatiser la RPA par intégration dans un système microfluidique miniaturisé centripète. Une collaboration avec des experts en génies et en matériaux a permis de générer un dispositif microfluidique appelé *blade* ainsi de l'instrument impliqué dans l'opération

des différentes tâches mécanistiques. Ces résultats préliminaires suggèrent qu'il sera important d'offrir un système automatisé complet applicable au chevet du patient. Par conséquence, il sera possible d'exécuter une analyse complète des agents infectieux en moins d'une heure sans le besoin des procédures complexes de préparation et de transport des échantillons cliniques ni le recours à du personnel qualifié.

Abstract

This dissertation consists of an exhaustive study on an emerging technology for isothermal amplification of nucleic acids called recombinase polymerase amplification (RPA). The introduction of this thesis is a detailed description of the RPA. This review documents and discusses the various applications of this technology by pointing to the current knowledge about RPA for diagnostic applications. Despite the complex composition of RPA (6 to 7 proteins in the same reaction mixture), the latter was shown to be rapid (generating results in <20 min), specific and sensitive (detecting few target genome copies), and applied widely in different fields. Based on these advantages, we assume that RPA has a flexibility allowing it to be used for the rapid diagnosis of infectious diseases thus reducing time-to-result to less than an hour. Consequently, it will be possible to integrate RPA in microfluidic platforms providing a lab-on chip system. The first part of this doctoral project generated additional guidelines for RPA primers/probes design to develop specific RPA diagnostic assays. Second, we developed an RPA diagnostic test for the detection of group B streptococci, responsible for sepsis and meningitis in newborns. This assay was the first to evaluate RPA with human clinical samples. This diagnostic test was compared to a reference method, the polymerase chain reaction (PCR). This demonstration with clinical samples served to carry out the final objective of this project that was to automate RPA in a miniaturized microfluidic centripetal system. Collaboration with engineers and experts in materials has generated the microfluidic device called "blade" and the instrument involved in the operation of various mechanistic tasks. These preliminary results suggested that it will be important to provide an automated system applicable at bedside. Consequently, it will be possible to perform a complete analysis of infectious agents in less than an hour without the need for complex procedures for the preparation and transport of clinical specimens or the assistance of qualified personnel.

Table of contents

List of tables

List of figures

List of abbreviations

AFU, arbitrary fluorescence units
ASSURED, accurate; specific; sensitive; user-friendly; robust; equipment-free; delivered to those in need
CDC, centers for disease control and prevention
cfb, CAMP-factor B
CLIA, Clinical Laboratory Improvement Amendments
CPA, cross-priming amplification
Ct, cycle threshold
dsDNA, double strand DNA
DVD, digital versatile discs
ELISA, enzyme-linked immunosorbant assay
HDA, helicase-dependent amplification
HIV, Human immunodeficiency virus
IC_{RPA}, RPA assay internal control
ISAD, isothermal solid-phase amplification/detection
LAMP, loop-mediated amplification
LF-RPA, lateral flow RPA
LOD, limit of detection
MDA, multiple displacement amplification
MuLV, Murine leukemia virus
NASBA, nucleic acid sequence-based amplification
NEAR, nicking-enzyme amplification reaction
NGS, Next Generation Sequencing
NINA, non-instrumented nucleic acid amplification
NPV, negative predictive value
PCR, polymerase chain reaction
POC, point-of-care
PPV, positive predictive value
RCA, rolling circle amplification
RPA, recombinase polymerase amplification
rtPCR, real-time PCR
RT-RPA, reverse transcriptase RPA
rt-RPA, real-time RPA
RMA, ribonuclease-mediated amplification
SAMRS, self-avoiding molecular recognition system
SCC*mec*, staphylococcal cassette chromosome *mec*
SDA, strand displacement amplification
SP, solid-phase
SSBs, single-strand binding proteins
***Taq* polymerase,** *Thermus aquaticus* polymerase
THF, tetrahydrofuran
TMA, transcription-mediated amplification
tuf, elongation factor Tu

List of abbreviations for bacterial and fungal species

A. caviae, *Aeromonas caviae*
B. atrophaeus, *Bacillus atrophaeus* subsp. *Globigii*
C. albicans, *Candida albicans*
C. difficile, *Clostridium difficile*
E. hoshiniae, *Edwardsiella hoshiniae*
GBS, group B Streptococcus
G. sanguinis, *Globicatella sanguinis*
M. avium, *Mycobacterium avium*
M. curtisii, *Mobiluncus curtisii* subsp. *holmesii*
M. gordonae, *Mycobacterium gordonae*
MRSA, methicillin-resistant *Staphylococcus aureus*
M. senegalense, *Mycobacterium senegalense*
M. septicum, *Mycobacterium septicum*
M. smegmatis, *Mycobacterium smegmatis*
M. tuberculosis, *Mycobacterium tuberculosis*
N. gonorrhoeae, *Neisseria gonorrhoeae*
P. aeruginosa, *Pseudomonas aeruginosa*
P. oryzihabitans, *Pseudomonas oryzihabitans*
S. agalactiae, *Streptococcus agalactiae*
S. arizonae, *Salmonella enterica* subsp. *arizonae*
S. aureus, *Staphylococcus aureus*
S. canis, *Streptococcus canis*
S. enterica, *Salmonella enterica* subsp. *enterica* Gallinarum
S. iniae, *Streptococcus iniae*
S. pyogenes, *Streptococcus pyogenes*
S. uberis, *Streptococcus uberis*

"A little knowledge that acts is worth infinitely more than much knowledge that is idle"

"Trust in dreams, for in them is hidden the gate to eternity"

"Faith is an oasis in the heart which will never be reached by the caravan of thinking"

Gibran Khalil Gibran (1883-1931)

To my country Lebanon and my beloved Family,

Acknowledgements

I owe the accomplishment of this thesis to the help and contribution of great persons that words are not enough to express my gratitude to every one of them.

Firstly, I thank my director Dr. Michel G. Bergeron for giving me this opportunity to conduct my PhD studies in his prestigious laboratory. Dr. Bergeron, you are an idol and an inspiration to me. I learned from you the determination and the devotion to dreams and ambitions. I also thank you for your support at many occasions during all these years.

This PhD project would not have succeeded without the mentoring of Dr. Gale Stewart. I thank you Gale for your advice and your patience that you offered pleasantly. I also thank you for being caring and a good listener at all time. Thank you for your generosity and for the sweet attentions that made working with you such a pleasure.

The progression of this project could not be obtained without the mentoring of Dr. Maurice Boissinot. Your contribution was very important for leading this work to its scientific maturity. I thank you for your encouragements and your unlimited stimulations throughout these years.

Working in the laboratory was fun and exciting because of a great team, the ***Équipe de Diagnostic Moléculaire*** **(EDM)** team. The list is long but I won't miss the opportunity to thank every one of you who made my experience memorable. I will start to thank Dr. Luc Bissonnette for his sharp vision in correcting scientific documents. Your advices were of great utility. I also thank Dr. Ann Huletsky for always being ready for help and for your positive attitude. Our sweet and friendly talks were very remarkable and lovely. The EDM team is formed of great persons who are dedicated to their work and who have amazing values and qualities.

My days, months, and years among you were painted with hard work and perseverance, and crowned with laughter and mutual appreciation. Therefore, I sincerely thank the present and past members of the EDM team.

This thesis project was in part in collaboration with experts in engineering and materials. I seize the opportunity to thank all the members who contributed to the accomplishment of this project. I thank members of the *Centre d'optique, photonique et laser* (COPL), David Béliveau-Viel and Denis Boudreau. Working with you was fun and very instructive. I also thank members of National Research Council Canada (NRC), Samuel Clabecq, Maxence Mounier, and Teodor Veres. Your contribution to the progression of the project was important and really appreciated.

A special thanks to the administration members of the *Centre de recherche en infectiologie* (CRI), Pierre Provencher, Guylaine Mercier, and Brigitte Lambert. I also thank Lynn Duchesneau, Nathalie Pagé, and Gina Racine for being kind and thoughtful. I also want to acknowledge the cooperation of Yvette Pouliot, member of the library services at the *Centre de Recherche du CHU de Québec*, *Centre Hospitalier de l'Université Laval (CHUL)* that was very useful during all these years of my PhD studies.

During those 7 years or so of my stay in Canada for my master and PhD studies, my adaptation and acclimatization to the *Québecoise* society were made possible because of an exceptional friend, Geneviève Filion. I am happy to have the occasion to express my gratitude to you and to your parents. All those years, you embraced me with your unconditional love, your great hospitality, and your limitless support. I thank you Lyne and Serge for considering me as a member of your family and for filling my experience in Canada at your sweet hometown, *La Tuque*, with unforgettable souvenirs. I also thank Carole, Phillippe, Louis-Philippe, and Mario for your friendship, and for the joyful moments that we lived together. All of

you have warmed up my heart throughout these years of long white winter in Canada.

At the end, the biggest and the warmest gratitude is to my family; to my parents, Kawkab Al Fidawi and Yaacoub Daher who always believed in me and motivated me and endured all these years of separation. Your unconditional love, your limitless care and support, and your understanding were my strength; filling my heart with confidence and perseverance. My brothers Raja, Robert, and Rami, you were and always be my guardian angels. My sisters Roula, Rima, and Rouba, my beautiful roses, I thank you for your infinite tenderness and your trustworthiness. My precious family, I dedicate this thesis to you!

Foreword

This doctoral thesis comprises two published articles that are incorporated in their post-print version.

Article 1 constitutes chapter III: Rana K. Daher, Gale Stewart, Maurice Boissinot, Dominique K. Boudreau, and Michel G. Bergeron. Influence of sequence mismatches on the specificity of recombinase polymerase amplification technology. Molecular and cellular Probes 2015; v. 29 (2), p.116-121. This article is licensed under a Creative Commons Attribution-Non Commercial-No Derivatives 4.0 International License (CC-BY-NC-ND).
Published online: http://www.ncbi.nlm.nih.gov/pubmed/25481659.

Article 2 constitutes chapter IV: Rana K. Daher, Gale Stewart, Maurice Boissinot, and Michel G. Bergeron. Isothermal Recombinase Polymerase Amplification Assay Applied to the Detection of Group B Streptococci in Vaginal/Anal Samples. Clinical Chemistry 2014; v. 60, p.660-666. This article is reproduced in this thesis with permission from the American Association for Clinical Chemistry.
Published online: http://www.ncbi.nlm.nih.gov/pubmed/24463560.

Authors' contribution for each article is detailed in a separate foreword section at the beginning of each chapter. Figures and tables numbers were modified for uniformity. For example, figure 1.3 designates *figure 3 of chapter I* and table 1.3A designates *table 3A of chapter I*.

I. Introduction

Recombinase polymerase amplification (RPA) technology

1.1 Isothermal nucleic acid amplification technologies

Specific amplification of nucleic acid sequence is a key process for pathogen detection in molecular diagnostics. Since the availability of thermostable *Taq* polymerase, polymerase chain reaction (PCR) has been the workhorse amplification technology in laboratories worldwide. However, PCR requirements for a thermal cycler limits its application to centralized laboratories and makes its integration into miniaturized lab-on-a-chip devices challenging (de Paz, Brotons *et al.* 2014).

During the last decade, there was a remarkable increase in the development or adaptation of novel and existing isothermal-dependent amplification technologies. Isothermal amplification technologies offer some advantages compared to PCR. First, the reaction occurs at a single temperature, hence obviating the need for thermal cyclers (Li and Macdonald 2015). Second, these technologies also showed good tolerance to PCR-inhibitors commonly found in clinical samples (de Paz, Brotons *et al.* 2014). The diversity of isothermal technologies has promoted their application in research and development as well as clinical fields (e.g. first isothermal CLIA waiver Alere i Influenza A&B assay, (Bell, Bonner *et al.* 2014)). More than a dozen isothermal technologies exist differing in their respective enzymatic mechanisms and are reviewed elsewhere (de Paz, Brotons *et al.* 2014; Yan, Zhou *et al.* 2014; Li and Macdonald 2015). A short list of isothermal technologies will also be reviewed and discussed in chapter VI of this thesis (table 6.1). However in this chapter, we simply describe some of the commonest isothermal technologies.

First, nucleic acid sequence-based amplification (NASBA) has been applied primarily for the amplification of RNA targets employing 3 key enzymes (i.e. Avian Myeloblastosis Virus reverse transcriptase, RNase H, and T7 DNA dependent RNA polymerase) and 2 primers all functioning at 41°C. On the other hand, NASBA is not truly isothermal necessitating an initial priming step at 65°C and a heat denaturation step at 95°C (when working with DNA). Consequently, enzymes are added separately due to their thermolability. In addition, NASBA is limited to short amplicon sequences (120 - 250 bp) (Gracias and McKillip 2007).

Second, loop-mediated isothermal amplification (LAMP) is an isothermal technology depending on the strand-displacement activity of a polymerase enzyme. Four to six primers recognising 6-8 different regions in the target sequence are required rendering the amplification process highly specific. Up to 10^9 copies of DNA are generated in 1 hour at 60-65°C. Despite the robustness of LAMP in terms of tolerance to common PCR inhibitors, speed of reaction, and amplification efficiency, it also requires an initial denaturation step at 95°C and shows some complexity in multiplexing due LAMP primer design as well as the production of non-specific amplification products (Notomi, Okayama *et al.* 2000).

Amplification of nucleic acids requires separation of the DNA double strands that is accomplished in PCR by the thermal cycle. For isothermal technologies, while certain techniques still require an initial heat denaturation step at 95°C, others rely on enzymes to separate the double strands. For instance, helicase-dependant amplification (HDA) employs helicases along with single-strand binding proteins (SSBs) to separate double stranded DNA (dsDNA) target and prevent its re-annealing. Elongation of hybridized primers is executed by DNA polymerase, functioning at 37°C. Improvements of HDA technology had enhanced the coordination of helicase with DNA polymerase increasing its speed and processivity, and allowing amplification of long nucleotides sequences up to 2.3 kb in 2 hours. However, in its improved format, HDA works at higher temperature 60-65°C that is translated in more complex instrumentation (Vincent, Xu *et al.* 2004; Gill, Amini *et al.* 2007).

Recombinase polymerase amplification (RPA) is another enzyme-based isothermal amplification technology that earned the spotlight of many scientists in the last 4 years (figure1.1). This recombinase-dependent isothermal amplification entails two primers and one probe (optional) with simple design requirements. For DNA unwinding and primer annealing, RPA uses recombinase enzymes with accessory proteins. It has a fast reaction scheme (*i.e.* <20 min) and a true isothermal profile without additional temperature fluctuation. RPA has high specificity and efficiency (10^4–fold amplification in 10 min) (Piepenburg, Williams *et al.* 2006). RPA reagent components offer different

detection methods and are available in lyophilized formats facilitating its applications in diagnostics.

Consequently, RPA technology has been widely used in different research fields resulting in a remarkable increase in publications; more than 60 written works are now available on PubMed (figure 1.1).

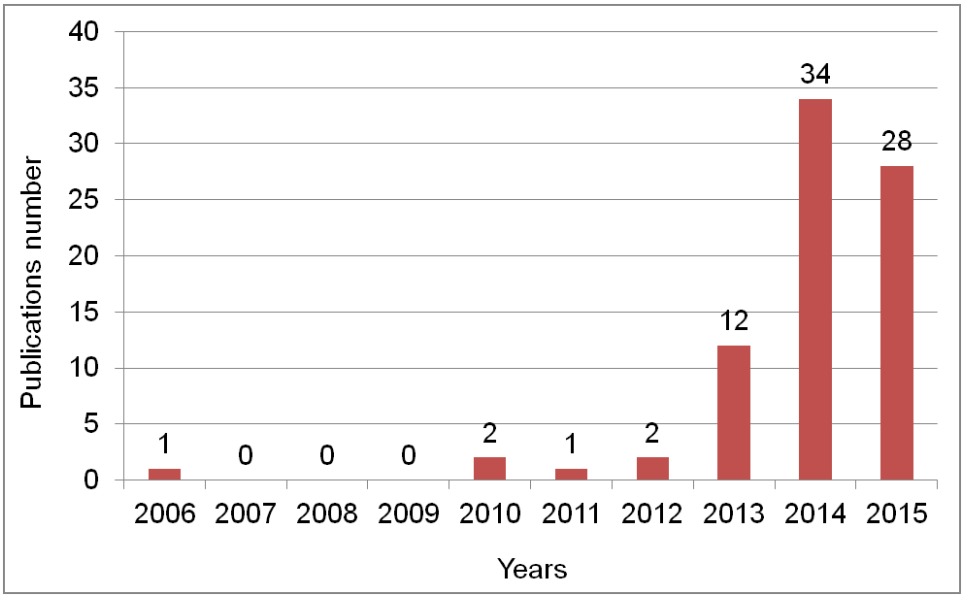

Figure 1.1. RPA publications profile

After the first published work in 2006, there was a gap of 3 years before that other publications start to appear and reach a peak in 2014.

1.2 RPA technology description

In 2006, Piepenburg *et al.* introduced for the first time RPA technology (Piepenburg, Williams *et al.* 2006). RPA comprises three key proteins to substitute the usual PCR heat denaturation: recombinase protein (*E.coli* RecA), ssDNA binding protein (SSB), and DNA polymerase with strand-displacing activity (*Sau* polymerase). These proteins are central components of *in vivo* DNA repair and homologous recombination processes. Accessory proteins and cofactors also support the RPA reaction process such as T4 UvsY protein (recombinase loading factor), crowding agent (polyethylene glycol), phosphocreatine, and creatine kinase (ATP-fuel system). This composition is used in RPA kits but another composition is described in the original paper of Piepenburg. For instance, recombinase RecA could be replaced by T4 UvsX protein, SSB protein by T4 gp32, *Sau* polymerase by *Bsu* polymerase, and crowding agent PEG by Carbowax20M (Piepenburg, Williams *et al.* 2006).

In RPA, the recombinases assisted with the loading factor form a nucleoprotein filament with primers and probes (single strand oligonucleotides).This filament scans the dsDNA target searching for homologous sequences. Once homology is found, the filament invades the dsDNA forming a D-loop structure that is a local separation of DNA strands stabilized by SSBs (figure 1.2). ATP-hydrolysis induces recombinase disassembly from the nucleoprotein filament thus allowing primer annealing and elongation by strand-displacing polymerase enzyme. The generation of 2 duplexes is used for another round of RPA cycle. Consequently, an exponential amplification is accomplished by repetition of RPA cycle which is described as self-perpetuating (Piepenburg, Williams *et al.* 2014).

Typically, RPA reactions are executed in 5 – 20 min depending on the starting template copies and amplicon size (Hill-Cawthorne, Hudson *et al.* 2014). Additionally, it is possible with RPA to detect RNA targets by incorporating a reverse transcriptase to the RPA reagent components. It is a one-step reverse transcriptase RPA (RT-RPA) mechanism functioning constantly at one temperature (40-42°C, TwisAmp® exo or basic RT kits, respectively) (TwistDx 2015c; TwistDx 2015d).

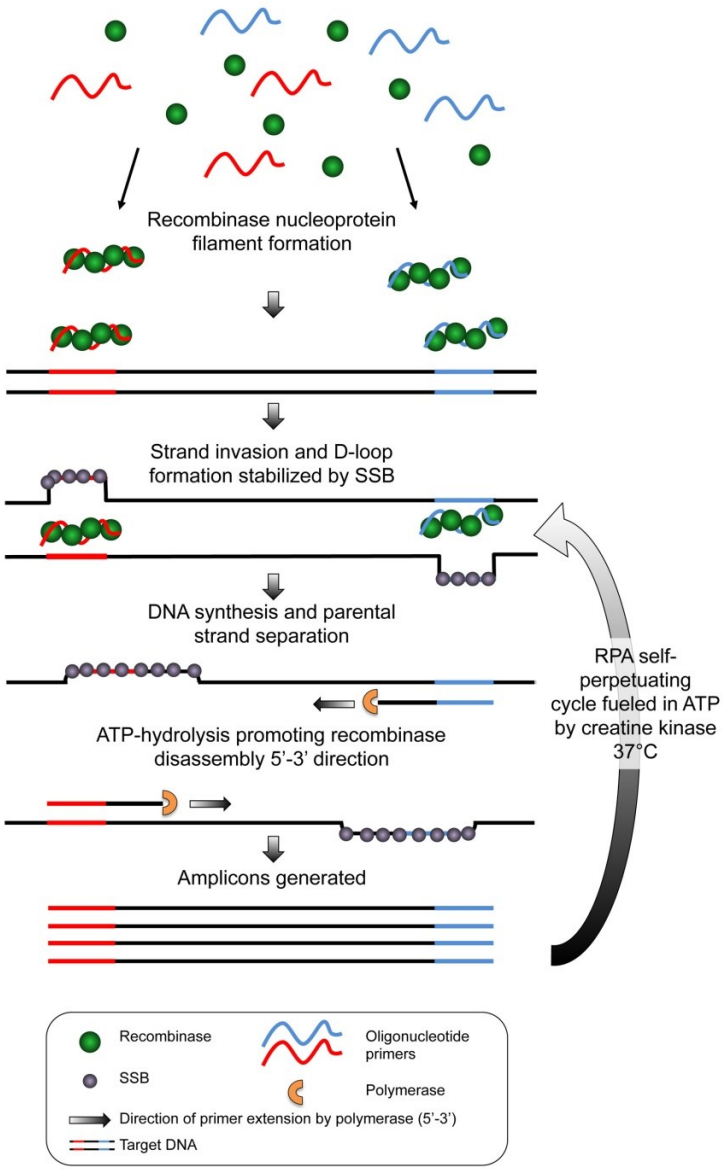

Figure 1.2. RPA Cycle

The three core proteins, recombinase, single-strand DNA binding protein (SSB) and strand-displacing polymerase enable exponential DNA amplification without the need for thermal cycling or an initial chemical or thermal melting step. The complete reaction is performed at a single temperature from 25°C-42°C depending on RPA reagent formulations.

1.3 RPA commercial kits

RPA technology is commercialised for research use only (TwistDx, Inc.) in different kit formats for either DNA or RNA amplification offering various detection systems such as agarose gel electrophoresis, lateral flow strips or real-time fluorescent probes (table 1.1) (Piepenburg, Williams *et al.* 2014). A typical RPA kit comprises in individual tubes 1x buffer solution, magnesium acetate solution (280 mM), lyophilized RPA reagents pellets, and controls (positive control DNA and positive control primers and/or probe). For certain applications, it is possible to incorporate target-specific RPA primers/probe to the lyophilized pellet as shown in table 1.1 such as kits for *Salmonella*, *Listeria*, *Campylobacter*, and Red Snapper (Fish species) detection. RPA kits are stored for up to 6 months at -20°C before use. The RPA experimental protocol is illustrated in figure 1.3 and comprises 5 stages. The first 4 stages necessitate simple hands-on manipulation however the last stage could avoid further manual manipulations if the real-time RPA format is used.

Table 1.1. RPA commercial kits

Kits (TwistAmp®)[1]	Target type	Incubation (°C)	Detection Probe	Post-amplification purification	Detection system[2]	Specific application kits
Basic	DNA	37-39	No	Yes	AG	-
Basic RT	RNA	40-42	No	Yes	AG	-
exo	DNA	37-39	Yes	No	rt	*Salmonella*, *Listeria monocytogenes*, *Campylobacter*[3]
exo RT	RNA	40-42	Yes	No	rt	-
fpg	DNA	37-39	Yes	Yes (only for AG detection)	rt/AG	-
nfo	DNA	37-39	Yes	Yes (only for AG detection)	LF/rt/AG	*Salmonella*, Red Snapper[4]

[1] RT = reverse transcription; exo = exonuclease III real-time detection format; fpg = Fpg nuclease real-time detection format; nfo = Nfo nuclease lateral-flow detection format.
[2] AG = agarose gel electrophoresis; rt = real-time; LF = lateral flow.
[3] kits for food safety and ID analysis: TwistGlow® *Salmonella*, TwistAmp® exo+*ListeriaM*, and TwistAmp® exo+*Campylobacter*.
[4] kits for food safety and ID analysis: TwistFlow® *Salmonella* and TwistFlow® Red Snapper (Fish species identification).

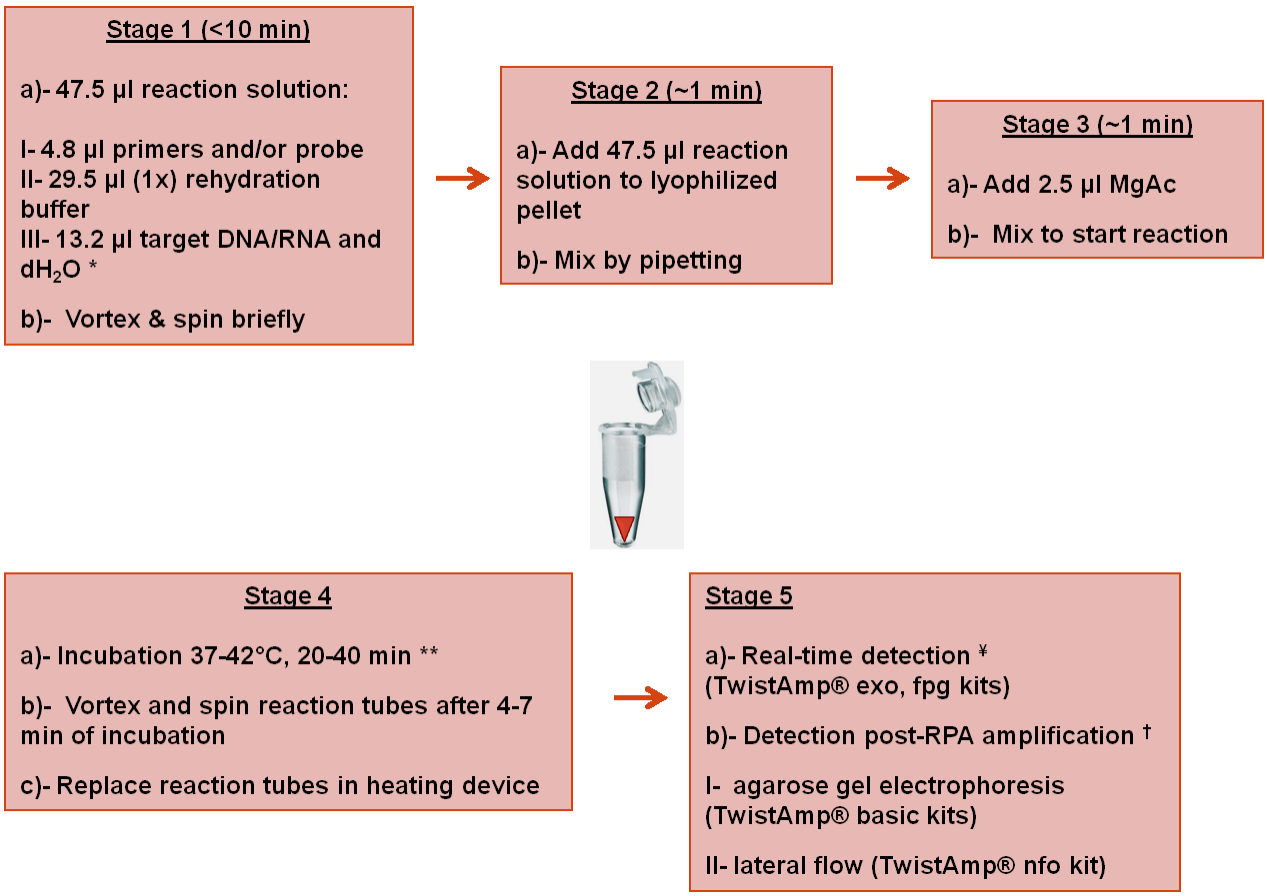

Figure 1.3. RPA experimental protocol

First, a reaction solution containing target nucleic acid is formed and added directly to one lyophilized RPA reagent pellet followed by a brief mixing and centrifugation. (* The time required for reaction solution preparation does not include sample preparation. When sample preparation is considered the time will be >10 min and depends on sample preparation procedure itself. When RNA is amplified the buffer solution can preferably contain RNase Inhibitor). Lastly, magnesium acetate solution is added to start the reaction. ** Incubation is performed at one temperature raging between 37°C-42°C depending on RPA kit used (table 1.1). After incubation, the detection of RPA products can be either in real-time or end-point. ¥ With real-time detection, results are obtained during the incubation stage (# 4) in <10 min for strong positive samples. † With post-RPA detection, when agarose gel electrophoresis is used, RPA amplicons must first be purified then migrated on a gel taking >1 hour for results generation. With lateral-flow detection, RPA amplicons can be used directly without purification generating results on the dipstick strip within 5 min.

1.4 RPA primers and probes design

According to the manufacturer's guidelines, RPA primers should be 30 to 35 bases long, longer primers (>45 bases) are not recommended. Long tracks of one particular nucleotide or a large number of small repeats should be avoided. High or low GC content (>70% or <30%, respectively) must also be avoided. RPA can amplify long sequences up to 1.5 kb however best performance can be achieved with shorter amplicons of 80-400 bp (100-200 bp, optimal). In addition, there are no Tm requirements for RPA primers and probes since primer annealing and elongation are enzyme-mediated processes and not thermal (TwistDx 2015d).

For real-time detection, two probes RPA-exo or fpg are used as it appears that conventional probes could not be used in RPA reactions (TwistDx). Exo probe is used with TwistAmp® exo and exo RT kits. This probe is a long nucleotide sequence between 46 and 52 bases bearing an internal abasic site (e.g. tetrahydrofuran (THF)) located between a fluorophore (e.g. FAM or TAMRA) and a quencher (e.g. Black Hole Quencher 1 or Black Hole Quencher 2) with a blocked 3' end (e.g. 3' phosphate group or dideoxynucleotide). This abasic site is a substrate for *E.coli* exonuclease III that cleaves it after binding of the probe to the target sequence (Piepenburg and Armes 2011); thus separating the fluorophore from its quencher (figure 1.4). Fluorescence generation typically yields a detectable signal within 5-10 min during the RPA reaction (Hill-Cawthorne, Hudson *et al.* 2014). On the other hand, exonuclease cleavage generates a free 3'-end of the exo probe which is then extendable by the polymerase enzyme and can serve as a forward primer (Piepenburg, Williams *et al.* 2006). Fpg probe is used with TwistAmp® fpg kit. Compared to the exo probe, the fpg probe is shorter 32-35 bases and does not serve as a primer. It has a 5' quencher and the fluorophore attached to the ribose abasic site via a C-O-C linker, also dR-group at 5-6 bases downstream. The DNA glycosylase/lyase *E.coli* Fpg protein has a different catalytic mode than exonuclease III. It recognises and cleaves the dR-group while leaving two non-extendable strands (Piepenburg and Armes 2011) (figure 1.5).

For post-RPA lateral flow detection using TwistAmp® nfo Kit, LF-probe design requires a 5'-fluorophore tag (*i.e.* FAM) without a quencher. However, it still contains a THF

residue for Nfo endonuclease recognition and cleavage, thus serving as a primer similar to the exo probe (figure 1.6). Nfo endonuclease can therefore replace exonuclease III since both recognise the same substrate (*i.e.* THF residue). However, Nfo nuclease generates a slower signal and incomplete cleavage. This avoids amplicon degradation and allows detection by gel electrophoresis which is impossible with the Twist probe (Piepenburg and Armes 2011). For lateral flow assay, the reverse primer (opposing amplification primer) has a 5'-biotin label and forms with the labeled nfo probe a double labelled amplicon which is then captured with species-specific anti-FAM antibody coupled to gold nanoparticles. Another immobilized antibody captures biotin forming thus the detection line on commercially available strips (MGHD 1, TwistDx, UK). A control line with immobilized anti-species antibodies serves as an assay control (figure 1.6).

By analyzing a total of 204 RPA primers and 64 probes (exo (75%), fpg (14%), and nfo (11%) probes) sequences originating from 40 (~70%) published RPA articles, we observed that the above mentioned guidelines for RPA primers/probes design were not always respected. For instance, 7% of RPA primers exceeded 35 bases (36-45 bases long) and 9% were below 30 bases long, short sequences of 20-23 bases were used especially in studies where primers served as capture probes at the same time (Santiago-Felipe, Tortajada-Genaro *et al.* 2014a; Santiago-Felipe, Tortajada-Genaro *et al.* 2015). Ten percent of primer sequences had GC content <30% and 3% had GC content >65%. For published exo probe sequences, 17% of exo probes were shorter than the required length (34-44 bases instead of 46-52 bases) and 10% were of 53 and 58 bases long. A small proportion (6%) of exo probes had GC content below 30%. Most of the works that were considered in our analysis used the exo probe real-time detection method (75%) rather than fpg (14%) or nfo (11%) probes. Therefore, we considered that discrepant data for fpg and nfo probes were not as representative as those for exo probes and thus are not presented herein.

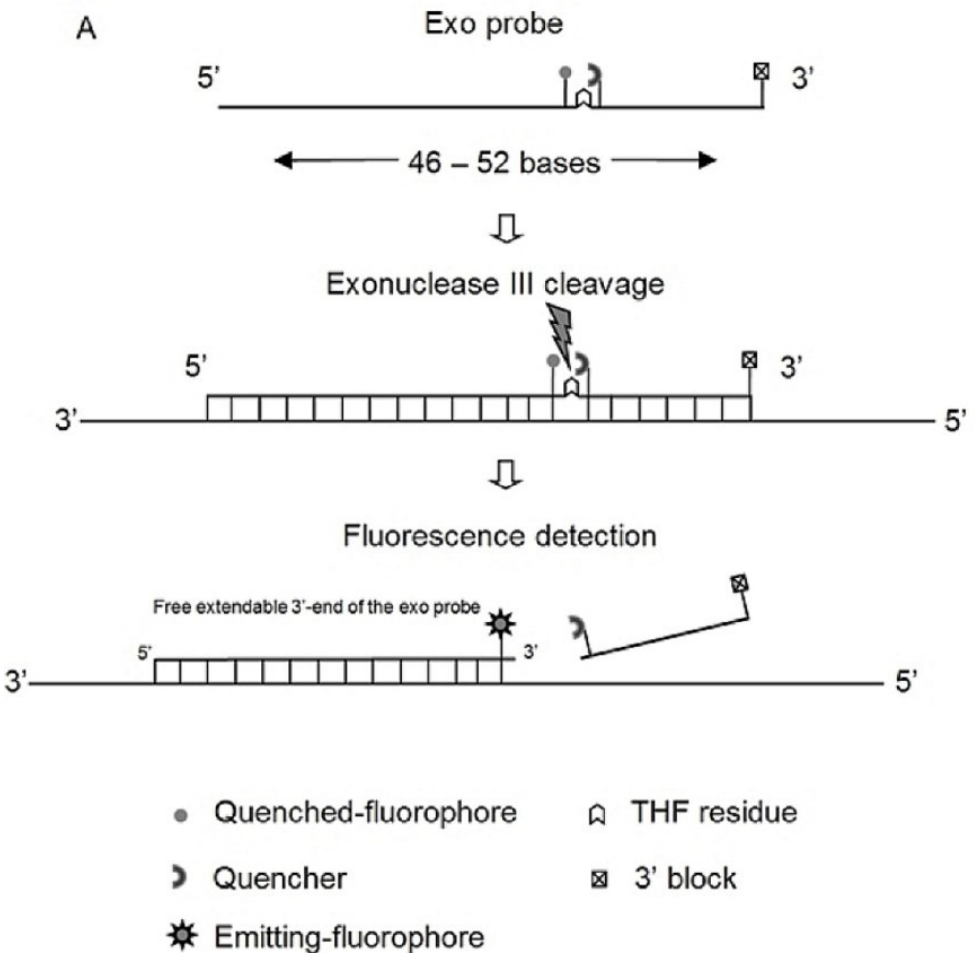

Figure 1.4. Real-time RPA detection with exo probe

Only when RPA-exo probe binds to the target DNA, exonuclease III enzyme recognizes its substrate and cuts the internal THF residue thus liberating the internal fluorophore from its quencher.

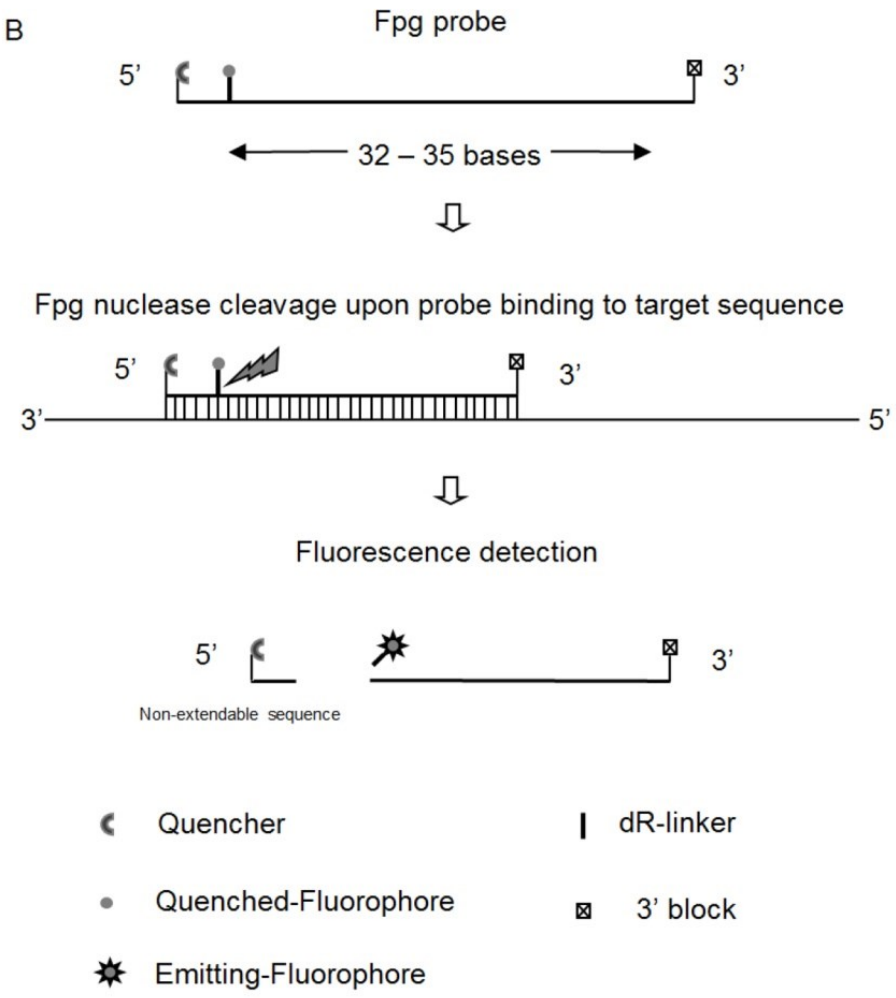

Figure 1.5. Real-time RPA detection with fpg probe

Only when RPA-fpg probe binds to the target DNA, Fpg nuclease recognizes the dR-group and cuts its substrate thus liberating the fluorophore from its quencher.

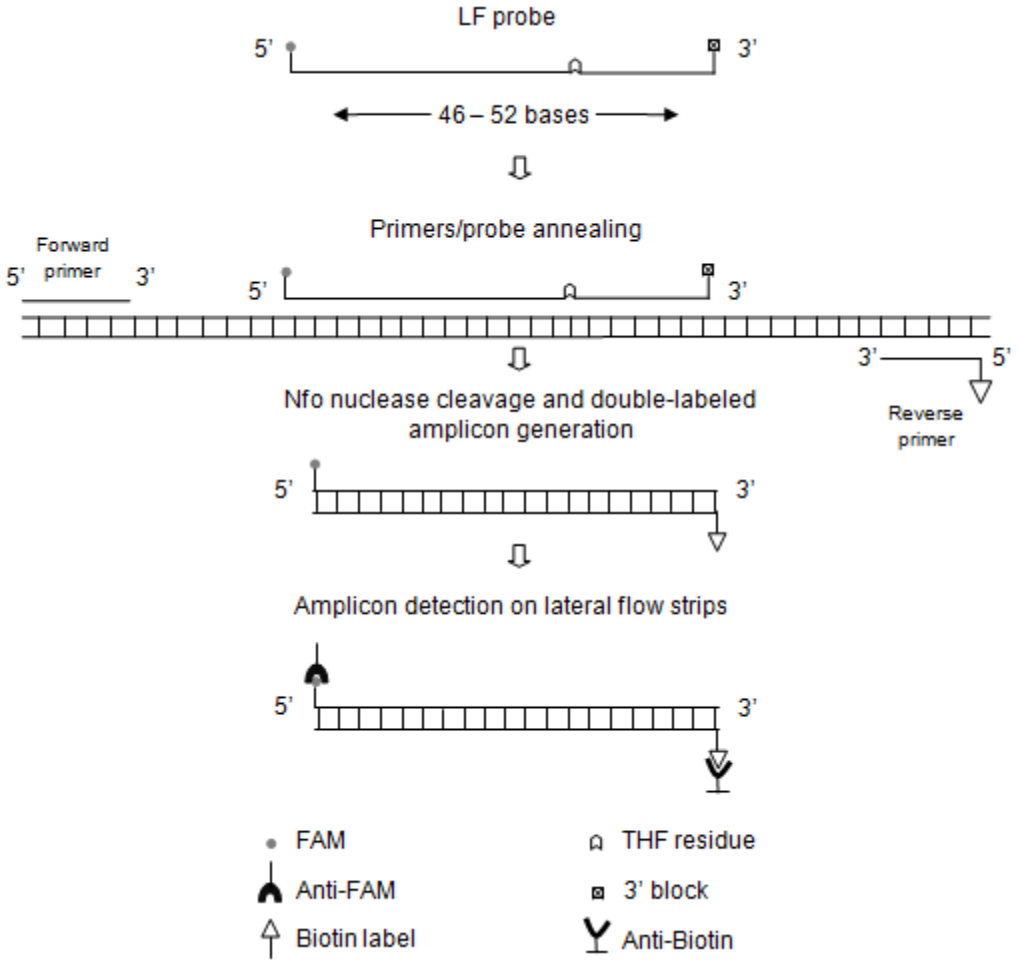

Figure 1.6. Lateral flow post-RPA detection

RPA-LF probe is 5'-tagged with FAM which upon Nfo endonuclease cleavage generates along with the 5'-biotin labeled reverse primer an amplicon that has both FAM and Biotin tags. Amplicon detection is accomplished by capture of both tags with anti-FAM and anti-Biotin antibodies generating a visual colored line on LF strips.

1.5 RPA applications diversity

The RPA protocol (figure 1.3) with its various commercial kit formats (table 1.1) has been adapted for the detection of different pathogen including bacteria (Euler, Wang *et al.* 2012b; Euler, Wang *et al.* 2013; Ahmed, van der Linden *et al.* 2014; Boyle, McNerney *et al.* 2014; Daher, Stewart *et al.* 2014; del Río, Yehia Adly *et al.* 2014; Hill-Cawthorne, Hudson *et al.* 2014; Kersting, Rausch *et al.* 2014a; Kim, Park *et al.* 2014; Krolov, Frolova *et al.* 2014; Murinda, Ibekwe *et al.* 2014; Tsaloglou, Watson *et al.* 2015), viruses (Euler, Wang *et al.* 2012a; Abd El Wahed, El-Deeb *et al.* 2013; Abd El Wahed, Patel *et al.* 2013; Amer, Abd El Wahed *et al.* 2013; Escadafal, Paweska *et al.* 2013; Aebischer, Wernike *et al.* 2014; Mekuria, Zhang *et al.* 2014; Xia, Yu *et al.* 2014; Zhang, Ravelonandro *et al.* 2014; Teoh, Sam *et al.* 2015), fungi (Sakai, Trabasso *et al.* 2014), parasites (Crannell, Castellanos-Gonzalez *et al.* 2014; Kersting, Rausch *et al.* 2014b), as well as genetically modified organisms (Xu, Li *et al.* 2014a; Xu, Li *et al.* 2014b) and genetic alterations observed in cancer cells (Loo, Lau *et al.* 2013; Shin, Perera *et al.* 2013). It was also used for early infant diagnosis of HIV in low-resource settings (Boyle, Lehman *et al.* 2013; Crannell, Rohrman *et al.* 2014b). Tables 1.2, 1.3A and 3B illustrate various applications of RPA for RNA and DNA pathogens. The detection limit and the detection time varied between assays. For instance, 10 Dengue virus RNA copies were detected in <20 min while 16 Sigma virus RNA copies were detected in 4 min (table 1.2). For DNA pathogens, few *M. tuberculosis* and *Leptospira* DNA (1-2 DNA copies) were detected in 20-25 min while 98 *S. agalactiae* DNA copies were detected in 9 min (table 1.3A); suggesting that RPA efficiency could be dependent on target sequence, amplicon size, and type of biological sample used.

1.5.1 RNA pathogens

For RNA pathogens, using MuLV RT of TwistAmp® exo RT kit, Teoh and colleagues have implemented RT-RPA in a multiplex assay for the simultaneous screening of 4 dengue virus serotypes using 3 primers and 1 exo-probe (Teoh, Sam *et al.* 2015). This RT-RPA assay was performed from various biological samples including but not restricted to: spiked plasma, serum, blood, saliva demonstrating the tolerance of RT-

RPA to common inhibitors. However, it is noteworthy that the addition of RNAse inhibitor into RT-RPA reagents (as recommended by manufacturer) could play a role in RNA stability. This RT-RPA assay was able to detect as low as 10 target molecules in <20 min (Teoh, Sam *et al.* 2015) (table 1.2). Similar results were obtained in our hands. We determined the limit of detection (LOD) of MuLV RT assay (TwistAmp® exo RT kit) in amplifying RNA transcripts of Influenza A virus with and without the addition of a pool of nasopharyngeal swabs (a combination of different nasopharyngeal specimens negative for Influenza A virus) into RT-RPA mix. We obtained an LOD of 8.77 RNA copies ± 2.24 (95%CI) versus 13.5 RNA copies ± 6.6 (95%CI) in the presence and absence of the pool respectively. In both cases, 5 copies of RNA transcript were detected in <20 min thus correlating with the work of Teoh and colleagues (Appendix 1, table 1.4).

On the other hand, most of published work (86%) (table 1.2) used TwistAmp® exo kit with the addition of Transcriptor reverse transcriptase (Roche,Mannheim, Germany) (Abd El Wahed, El-Deeb *et al.* 2013; Abd El Wahed, Patel *et al.* 2013; Amer, Abd El Wahed *et al.* 2013; Euler, Wang *et al.* 2013; Aebischer, Wernike *et al.* 2014; Mekuria, Zhang *et al.* 2014). These studies were inspired from the work of Euler *et al*. who demonstrated that Transcriptor RT works better than MuLV RT (TwistDx, Babraham, UK) and Sensiscript RT (Qiagen, Hilden, Germany) when used with the TwistAmp® exo kit in an assay for Rift Valley fever virus screening (Euler, Wang *et al.* 2012a). When attempting to compare the performance of MuLV RT to Transcriptor RT, we obtained similar results for both enzymes; 10^4 copies of Influenza A RNA transcript were detected in 3.5 min with MuLV RT compared to 5 min with Transcriptor RT with similar fluorescence signal intensity (Appendix 1, figures 1.7A&B).

1.5.2 DNA pathogens

For DNA pathogens, researchers worked with TwistAmp® basic (9.5%), nfo (28.6%) and mostly with TwistAmp® exo kit (61.9%) (tables 1.3A and 3B). It was also demonstrated that the TwistAmp fpg probe had lower sensitivities (10^4 target molecules

detected) than the TwistAmp exo probe assays (10 target molecules detected) suggesting that Fpg nuclease has slower kinetics in real-time detection than that of exonuclease III (Euler, Wang *et al.* 2013). This could explain the wide use of the TwistAmp® exo kit.

Piepenburg *et al.* were the first to apply RPA for the detection of methicillin-resistant *Staphylococcus aureus (*MRSA) (Piepenburg, Williams *et al.* 2006). They developed a multiplex assay for the detection of 3 variants of the staphylococcal cassette chromosome *mec* (SCC*mec*) element. The multiplex comprised 4 primers and 3 probes including an internal control in a single RPA reaction. The proposed multiplex was able to detect 10 genomic copies of the 3 MRSA variants in <30 min in both real-time and lateral-flow detection formats (Piepenburg, Williams *et al.* 2006). In another study, Piepenburg and colleagues (Hill-Cawthorne, Hudson *et al.* 2014) presented a similar MRSA multiplex assay for the detection but not differentiation of 726 MRSA isolates from a single hospital. They demonstrated that molecular tests for MRSA, alone, cannot detect all MRSA isolates and that a proportion of 4% will remain undetected. This failure was demonstrated by genome sequencing methods to be due to recombination events within the SCC*mec* element that were not covered by common MRSA molecular assays (Hill-Cawthorne, Hudson *et al.* 2014).

1.5.3 RPA performance in presence of contaminants

RPA has demonstrated certain tolerance to common PCR inhibitors. It has been shown to function on various sample materials. For instance, RPA was able to function in the presence of up to 50 g/l of haemoglobin, 4% v/v of ethanol, 0.5 U of heparin, and serum (Kersting, Rausch *et al.* 2014b). However, RPA was inhibited when whole blood was used (Kersting, Rausch *et al.* 2014b). It was demonstrated that the concentration of background DNA present in whole blood samples was responsible of RPA inhibition especially with low target DNA concentration. For this reason, a lateral flow enrichment protocol was proposed enabling the detection of 10^4 copies of HIV-1 DNA in the presence of up to 14 µg of background DNA (Rohrman and Richards-Kortum 2015).

During this doctoral project, we had an objective to evaluate RPA with clinical samples particularly with vaginal/anal specimens for the screening of *Streptococcus agalactiae* (Daher, Stewart *et al.* 2014). The assay is detailed in chapter IV of this thesis.

Furthermore, RPA could be adapted to different detection methods such as solid-phase systems. Several studies have integrated it in miniaturised or equipment-free devices (Lutz, Weber *et al.* 2010; Shen, Davydova *et al.* 2011; Hakenberg, Hugle *et al.* 2012; Rohrman and Richards-Kortum 2012; Crannell, Rohrman *et al.* 2014a; del Río, Yehia Adly *et al.* 2014; Escadafal, Faye *et al.* 2014; Kersting, Rausch *et al.* 2014a; Kim, Park *et al.* 2014; Lee, Shin *et al.* 2014; Lillis, Lehman *et al.* 2014; Santiago-Felipe, Tortajada-Genaro *et al.* 2014a; Shin, Perera *et al.* 2015; Tsaloglou, Watson *et al.* 2015). For example, miniaturized semi-automated cartridges were applied for pathogens such as Yellow fever virus (Escadafal, Faye *et al.* 2014), *Mycobacterium tuberculosis* (Shin, Perera *et al.* 2015), and *Clostridium difficile* (Tsaloglou, Watson *et al.* 2015). In the following sections, we summarise RPA applications in different detection systems with emphasis on developed microfluidic platforms.

Table 1.2. Application of RPA for RNA pathogens

Pathogen	Biological samples	Analytical Sensitivity (copies)	Time-to-result (min)	Detection method[a]	Multiplex (#primers;#probes)[b]	Reference
Rift Valley fever virus	No	19	8	rt	No	(Euler, Wang *et al.* 2012a)
Rift Valley fever virus	Plasma[S]	19	7	rt	No	(Euler, Wang *et al.* 2013)
Bovine coronavirus	Fecal/Nasal Swabs	19	10-20	rt	No	(Amer, Abd El Wahed *et al.* 2013)
Middle East Respiratory Syndrome coronavirus	No	21	3-7	rt	No	(Abd El Wahed, Patel *et al.* 2013)
Foot-and-Mouth disease virus	Vesicular material/Saliva/ Serum/Blood/Nasal Swabs	100	4-10	rt	No	(Abd El Wahed, El-Deeb *et al.* 2013)
Schmallenberg virus	Blood/Serum	$6x10^3$	<10	rt	No	(Aebischer, Wernike *et al.* 2014)
Bovine Viral Diarrhea virus	Blood/Serum	$5x10^4$	<10	rt	No	(Aebischer, Wernike *et al.* 2014)
Ebola virus	Plasma[S]	21	7	rt	No	(Euler, Wang *et al.* 2013)
Sudan virus	Plasma[S]	17	8	rt	No	(Euler, Wang *et al.* 2013)
Marburg virus	Plasma[S]	21	8	rt	No	(Euler, Wang *et al.* 2013)
Sigma virus	Plasma[S]	16	4	rt	No	(Euler, Wang *et al.* 2013)
Dengue virus	Serum	10	<20	rt	Yes (3;1)	(Teoh, Sam *et al.* 2015)
Little cherry virus 2	Crude extracts	ND	25	LF	No	(Mekuria, Zhang *et al.* 2014)
Plum pox virus	Plant crude extracts	10^4	15-20	rt/LF	No	(Zhang, Ravelonandro *et al.* 2014)

[S] designates spiked biological sample.
ND = not determined, however the reported sensitivity was of 0.1 ng of pure RNA (Mekuria, Zhang *et al.* 2014).
[a] rt = real-time, TwistAmp® exo kit. LF = lateral flow, TwistAmp® nfo kit.
[b] No = simplex assay with only 2 primers and 1 probe.

Table 1.3A. Application of RPA for DNA pathogens

Pathogen	Biological samples	Analytical Sensitivity (copies)	Time-to-result (min)	Detection method[a]	Multiplex (#primers;#probes)[b]	Reference
Methicillin-resistant *Staphylococcus aureus*	No	<10	<30	rt/LF/AG	Yes (4;3)	(Piepenburg, Williams *et al.* 2006)
Francisella tularensis	Tissue	19	10	rt	No	(Euler, Wang *et al.* 2012b)
Francisella tularensis	Plasma[S]	19	10	rt	No	(Euler, Wang *et al.* 2013)
HIV-1 proviral	No	<10	20-30	rt/LF	No	(Boyle, Lehman *et al.* 2013)
Mycobacterium tuberculosis	Sputum/respiratory washes	~1*	<20	rt	No	(Boyle, McNerney *et al.* 2014)
Bacillus anthracis	Plasma[S]	16-778	7-8	rt	No	(Euler, Wang *et al.* 2013)
Yersinia pestis	Plasma[S]	16	8	rt	No	(Euler, Wang *et al.* 2013)
Variola virus	Plasma[S]	16	10	rt	No	(Euler, Wang *et al.* 2013)
Leptospira	Serum/Blood	<2	~25	rt	No	(Ahmed, van der Linden *et al.* 2014)
Chlamydia trachomatis	Urine	5-12	<20	LF	No	(Krolov, Frolova *et al.* 2014)
Shiga Toxin-producing *Escherichia coli*	No	ND	5-10	rt	No	(Murinda, Ibekwe *et al.* 2014)
Streptococcus agalactiae	Vaginal/Anal swabs	98	9	rt	Yes (4;2)	(Daher, Stewart *et al.* 2014)

* The reported sensitivity was of 6.5 fg which was estimated to contain the equivalent of a single bacterial cell (Boyle, McNerney *et al.* 2014).

[S] designates spiked biological sample.

ND = not determined, however the reported sensitivity was of 5-7 CFU/mL (Murinda, Ibekwe *et al.* 2014).

[a] rt = real-time, TwistAmp® exo kit. LF = lateral flow, TwistAmp® nfo kit.

[b] No = simplex assay with only 2 primers and 1 probe.

Table 1.3B. Application of RPA for DNA pathogens

Pathogen	Biological samples	Analytical Sensitivity (copies)	Time-to-result (min)	Detection method[a]	Multiplex[b]	Reference
Plasmodium falciparum	Whole Blood[S]/Serum[S]	~4*	<20	LF	No	(Kersting, Rausch *et al.* 2014b)
Giardia duodenalis	Extracted DNA from fresh stool	50	35	LF	No	(Crannell, Cabada *et al.* 2014)
Fungi	Fungal cells suspension	ND	~60	AG	No	(Sakai, Trabasso *et al.* 2014)
Shrimp White Spot Syndrome virus	Shrimp	10	6.41 ± 0.17	rt	No	(Xia, Yu *et al.* 2014)
***Penaeus stylirostris* densovirus**	Shrimp	100	35	LF	No	(Jaroenram and Owens 2014)
Infectious hypodermal and hematopoietic necrosis virus	Shrimp	4	7	rt	No	(Xia, Yu *et al.* 2015)

* the reported sensitivity was of 100 fg of genomic DNA which was estimated to contain the equivalent of 4 parasites per reaction (Kersting, Rausch *et al.* 2014b).
[S] designates spiked biological sample.
ND, not determined.
[a] rt = real-time, TwistAmp® exo kit. LF = lateral flow, TwistAmp® nfo kit. AG = agarose gel electrophoresis, TwistAmp® basic kit.
[b] No = simplex assay with only 2 primers and 1 probe.

1.6 Other RPA detection methods

To overcome non-specific RPA products (especially with low-quantity DNA target) and to allow multiplexing, RPA has been adapted into various end-point detection assays such as hybridization assays on microtitre plates (Santiago-Felipe, Tortajada-Genaro *et al.* 2014b), sandwich assays (Loo, Lau *et al.* 2013), and solid-phase platforms (SP-RPA) (Santiago-Felipe, Tortajada-Genaro *et al.* 2014a). On the other hand, Sharma *el al.* proposed another strategy to overcome non-specific RPA products. Their assay was based on incorporating few base-analogues into the 3'-end of typical RPA primers. These base-analogues were defined as self-avoiding molecular recognition system (SAMRS) and could not interact with each other thus avoiding the formation of primer-dimers (Sharma, Hoshika *et al.* 2014). Accordingly, we have investigated the specificity of RPA towards sequence mismatches in closely-related non-target genes which is depicted in chapter III (Daher, Stewart *et al.* 2015).

1.6.1 RPA-ELISA

RPA-ELISA consisted of detecting RPA amplicons with ELISA technology. This assay detected allergens, genetically modified organisms, pathogenic *Salmonella* sp. and *Cronobacter* sp., and fungal *Fusarium* sp. in a food safety analysis (Santiago-Felipe, Tortajada-Genaro *et al.* 2014b). Digoxigenin-labelled RPA amplicons were captured by specific probes attached to the microtitre plate through specific streptavidin-biotin conjugation. The detection occurred by visual color change due to enzyme-substrate reaction. Other isothermal technologies were also coupled with ELISA such as HDA (Gill, Amini *et al.* 2007) and LAMP (Ravan and Yazdanparast 2012). However, these other techniques work at high temperatures (60–65°C vs. 40°C for RPA-ELISA) and still require an initial heat denaturation step at 95°C (*i.e.* HDA) or complex primer design (*i.e.* LAMP) (Santiago-Felipe, Tortajada-Genaro *et al.* 2014b). In addition, RPA-ELISA showed comparable performance to PCR-ELISA yet with a simpler operation system. On the other hand, hybridisation assays as with ELISA are fastidious and demand several

optimisations (e.g. probes and antibody concentrations, hybridisation temperature and time or reaction volume). Furthermore, they typically involve multiple steps of amplicon treatment taking at least 90 minutes until signal detection (Santiago-Felipe, Tortajada-Genaro *et al.* 2014b).

1.6.2 RPA sandwich assay

RPA sandwich assay was developed for anti-cancer drug screening. In this assay, a single-stranded DNA named aptamer is used in a sandwich assay (antibody-antigen reaction) to specifically detect cytochrome-c, a common reporter of cancer cell death after typical chemotherapy (Loo, Lau *et al.* 2013). This work showed good specificity (owing to aptamer incorporation) and generated quantitative analysis (aptamer signal strength is an indicator for target molecules). In addition, RPA incorporation permitted the whole procedure to be accomplished in 3 hours compared to 8 hours for conventional western blot tests. This demonstrated the gain of speed when using RPA.

1.6.3 Solid-phase RPA

For SP-RPA detection platforms, different surfaces were used such as digital versatile disc (DVD) (Santiago-Felipe, Tortajada-Genaro *et al.* 2014a), silicon microring resonance (Shin, Perera *et al.* 2013), and epoxysilane glass slides (Kersting, Rausch *et al.* 2014a). The advantages of these platforms are in improving both specificity and sensitivity while offering low-cost, portable and robust sensors that allow multiplexing.

In SP-RPA on a DVD surface, hemi nested amplification took place within the first amplification product since the attached reverse primers differed from those in solution. Reverse primers were immobilised by streptavidin-biotin conjugation. The detection was performed with an optical reader such as a laser drive and the incubation was done in a conventional oven. The assay was developed for the

simultaneous detection of 3 *Salmonella* genes; two universal and one for species subtyping. The triplex assay was tested with 36 food and clinical samples (either spiked or raw) on a single DVD-drive in 40 min at single temperature of 37°C (Santiago-Felipe, Tortajada-Genaro *et al.* 2014a). The robustness of SP-RPA on DVD was also demonstrated in another study through the development of a duplex assay for the simultaneous detection of both *Salmonella* spp. and *Cronobacter* spp. in spiked milk samples. This assay was also compared to another isothermal technology, multiple displacement amplification (MDA) (Santiago-Felipe, Tortajada-Genaro *et al.* 2015). Both isothermal approaches showed comparable performance yet the RPA method demonstrated higher potential than MDA for diagnostic applications. Compared to RPA, the MDA method required an initial heat denaturation step at 95°C with the addition of dNTPs and polymerases separately. The MDA amplification is also performed at higher temperature (65°C), takes 4.5 hours for completion, and requires amplicon fragmentation prior to hybridisation. Although the proposed SP-RPA on DVD obviates the need for microfluidic devices it still has some limitations. In fact, several factors influence the efficiency and robustness of amplification on this platform and therefore must be considered. For instance, it was demonstrated that evaporation problems must be reduced, liquid volume, incubation temperature, concentration of bound and unbound primers, and the size of the solution and surface reaction products must be determined (Santiago-Felipe, Tortajada-Genaro *et al.* 2014a).

Furthermore, Shin and colleagues have developed a real-time, label-free SP-RPA called ISAD (isothermal solid-phase amplification/detection) for the screening of specific gene alterations in bladder cancer cells (Shin, Perera *et al.* 2013) and for the rapid detection of *Mycobacterium tuberculosis* (Shin, Perera *et al.* 2015). In their initial RPA-ISAD prototype, a primer that is specific for a cancer gene was covalently attached (via 5' end amine group modification) to a silicon microring surface and the detection of amplified products was possible by measuring a shift in the optical resonance wavelength. Subsequently, they validated the clinical utility of their prototype with sputum samples in a *M. tuberculosis* detection test. Despite the rapidity of the assay (within 20 min), the system lacked on-chip sample

preparation; DNA extraction from sputum samples was done off-chip and took 30 min (Shin, Perera *et al.* 2015). Others have applied ISAD-RPA for the detection of DNA-methylation in human cancers (Lee, Shin *et al.* 2014). This methylation-ISAD technique allowed a rapid time-to-result of 65 min compared to 5-24 hours for other methylation analysis (e.g. PCR), simple instrumentation (isothermal at 37°C) as well as reducing sample contamination (Lee, Shin *et al.* 2014). Despite the rapidity and simplicity offered by the current ISAD technology, it still lacks automation (*i.e.* several hands-on manipulations) and has yet to be validated on biological samples (*i.e.* proof-of-concept model with only genomic DNA).

Other RPA detection methods used epoxysilane glass slides bearing immobilized probes to specifically bind RPA amplicons. These applications allowed multiplexing where different pathogens (n= 4) were simultaneously detected (Kersting, Rausch *et al.* 2014a). This platform was run at 38°C and completed in <20 min with a detection limit of 10-100 colony-forming units (CFU). This assay offered the advantage of reducing non-specific products (especially when multiplexing), time, and costs while gaining more information from one sample analysis. However, this assay did not include an integrated on-chip sample preparation and was not validated with a representative number of biological samples (Kersting, Rausch *et al.* 2014a). Furthermore, several manual steps (performed by well-trained personnel) were required. Thus, the assay is not yet compatible for field tests.

1.7 Microfluidic platforms

The principal advantage behind microfluidic integration of nucleic acid amplification technologies is automation of all biological steps required for sample preparation, nucleic acid amplification, and detection. Automation results in shortening time-to-result, reducing sample and reagent volumes, minimising handling steps, and enhancing assay throughput and profitability (Lutz, Weber *et al.* 2010). Automation will render the molecular assay amenable for point-of-care (POC) and bring it near the patient. POC test attributes are summarized in the term "ASSURED" for affordable, sensitive, specific, user-friendly, rapid/robust, equipment-free, and delivered to the greatest need (Urdea, Penny *et al.* 2006). Consequently, RPA was employed in different settings with the goal of developing POC assays. RPA automation was the final objective of this thesis project and is described in chapter V.

In 2010, Lutz *et al.* were the first to integrate RPA exo in a centrifugal microfluidic cartridge containing prestored liquid and dry RPA reagents for the rapid detection of the *mecA* gene of *S. aureus* in 20 min (Lutz, Weber *et al.* 2010). The cartridge allowed conducting 30 parallel RPA reactions; the disc contained 6 chambers of 5 cavities and each chamber was prestored with lyophilized RPA reagents. The real-time detection of amplification signal was assured by incorporation of RPA-exo probes in the assay and fluorescence measurements. The procedure was performed with an adjusted Rotor-Gene instrument. This system was a proof-of-principle not validated with clinical samples. It detected as low as 2 copies of DNA target yet it lacked an on-chip sample preparation step (Lutz, Weber *et al.* 2010).

Parallel to this work, Kim and colleagues described a sample-to-answer cartridge similar to the previously described centrifugal microfluidic system of Lutz *et al*. (Lutz, Weber *et al.* 2010) but with the incorporation of a sample preparation via laser irradiation and a visual lateral-flow detection (Kim, Park *et al.* 2014). The disc consisted of 6 independent units processing 6 different RPA reactions, simultaneously. The workflow started with an initial manual loading step of sample/RPA solution (TwistFlow® *Salmonella* kit*)* but the rest of the procedure was

automated from sample preparation to detection. It was applied for the detection of *Salmonella* in spiked-milk samples and the full process was achieved in 30 min with a detection limit of 100 CFU/mL (Kim, Park *et al.* 2014). Although this fully integrated microfluidic cartridge offers a rapid and simple sample-to-answer procedure for pathogen screening, it still needs to be validated with other more complex biological samples while demonstrating both reproducibility and robustness.

Other types of microfluidic devices have also been used with RPA. Rohrman and colleagues have designed a paper and plastic platform for early infant HIV diagnosis (Rohrman and Richards-Kortum 2012). This platform differed from the previously mentioned microfluidic devices in that it was made with simple, inexpensive and user-friendly materials; no pumps are required but still it is able to perform many functions of microfluidic devices. The concept behind this invention was inspired from a previous work in which a microfluidic origami chip for bacterial DNA extraction was described (Govindarajan, Ramachandran *et al.* 2012). The paper and plastic device functioned after mixing reagents by simple diffusion by folding the platform. The detection of HIV DNA (PCR amplicon for HIV *gag* DNA as a target) was made by lateral flow strips with a reported detection limit of 10 copies within 15 min (Rohrman and Richards-Kortum 2012). In another study, they have applied this platform for the diagnosis of Cryptosporidiosis in stool samples (Crannell, Castellanos-Gonzalez *et al.* 2014). Despite its simplicity and cost-effectiveness however, there is a potential risk of contamination (Crannell, Castellanos-Gonzalez *et al.* 2014) since the handling is done in an open system; the detection method is not integrated with the amplification step and the rehydration solutions are manually dispensed. Second, the system lacks an integrated sample preparation procedure and further experiments need to be performed with fresh clinical samples.

Others have been interested in developing digital RPA assays on SlipChip platforms for DNA detection and quantification (Shen, Davydova *et al.* 2011; Tsaloglou, Watson *et al.* 2015). Knowing that the presence of divalent magnesium

ions is the initiator for RPA reaction (Piepenburg, Williams *et al.* 2006), it was mandatory to avoid potential false-positives by controlling the addition of magnesium into the RPA mixture either simultaneously or following the addition of a DNA target solution (Shen, Davydova *et al.* 2011). The SlipChip model offered the confinement of both the initiator and non-initiator, RPA basic reaction mixtures into separate compartments that can be assembled by slipping the plates which results in exchanging and homogenizing both reactions in parallel. The platform showed certain stability to temperature fluctuations and offered a quantitative analysis by endpoint fluorescence measurements (Shen, Davydova *et al.* 2011). However, their current platform required several manual steps increasing the risk of contamination (although it was not reported in their work) as well as it lacked the incorporation of on-chip sample preparation. Finally, with the actual read-out format, the system cannot be performed by untrained personnel, thus there are still further improvements to make this platform amenable to resource-limited areas. Another study applied a similar digital SlipChip platform but with a real-time detection of *Clostridium difficile* (toxin B) DNA using real-time RPA exo reagents (Tsaloglou, Watson *et al.* 2015). With real-time detection, the system offered better performance, especially since end-point analysis in RPA is prone to variability due to reaction kinetics which is poorly understood (Tsaloglou, Watson *et al.* 2015). Again, this platform requires further improvements and also lacked the integration of on-chip sample preparation.

Chapter

II. Project hypothesis & objectives

2.1 The challenge

Infectious diseases remain the major global health burden especially in developing countries (accounting for 95% toll of infections) (Hattersley, Greenman *et al.* 2013). Simple, rapid and cost-effective diagnostic tests are becoming a necessity. This requires translation of bringing bench-based assays into automated and miniaturized devices (Asiello and Baeumner 2011). Although PCR is the most common molecular method, however its application into miniaturized devices is challenging. The difficulty arises from the need of precise and fast temperature control during PCR cycles necessitating huge energy consumption (Asiello and Baeumner 2011).

Our laboratory is focused on developing rapid diagnostic tests that ultimately produce results in less than an hour allowing thus timely and appropriate medical intervention. We think that fast microbiological assays will not only reduce turnaround time but also will have an effect on costs and patient management. Long turnaround time increases the risk of disease transmission, morbidity, and mortality (Bissonnette, Chapdelaine *et al.* 2015). Therefore, we propose that the real challenge is to shorten successfully the molecular/biological process and automate the steps with microfluidics (Asiello and Baeumner 2011) to generate rapid microbiological tests that deliver results in the same time scale of other medical departments (*i.e.* radiology, biochemistry, and hematology) (Picard and Bergeron 2002). Figure 2.1 illustrates how I imagine the diagnostic cycle "clock" that can be shortened with rapid microbiological tests compared to standard culture-based tests that take >2 days. For instance, it will be possible with rapid tests to eliminate several undesirable and cumbersome steps thus generating results immediately (<1 hour) resulting in direct and appropriate medical intervention. Thus, proper use of antibiotics and less empirical treatment will result in better control of antibiotic resistant and hospital acquired infections.

As it was documented in the introduction of this thesis, recombinase polymerase amplification (RPA) technology offers many advantages compared to PCR and other isothermal amplification technologies. In contrast to certain isothermal

technologies, RPA is a true isothermal method that does not require an initial denaturation step. Second, its low operation temperature (25°C – 42°C) obviates the need for expensive instruments. Additionally, its speed of reaction (5 – 10 min for results), sensitivity (< 10 genome copies), multiplexing capability, and stability (up to one year full functionality of RPA dried reagents) made RPA more appealing for our research goals and an exciting subject for study and investigation, although we recognized its many challenges.

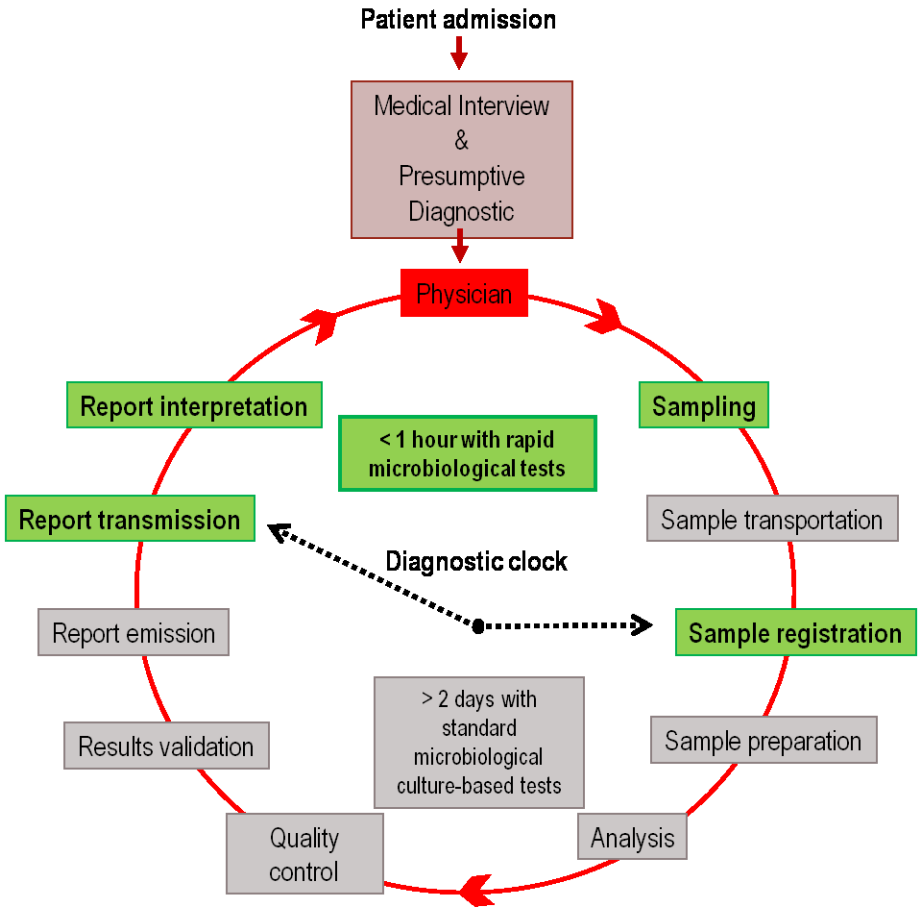

Figure 2.1. Diagnostic cycle clock of rapid microbiological tests

After patient admission, routine standard tests are performed. These include culture-based tests which are time consuming (>2 days). Automation would allow faster turnaround time (<1 hour, illustrated in green) while skipping the steps illustrated in grey.

2.2 Project hypothesis

Owing to aforementioned advantages, we think that RPA is a promising technology for the development of rapid and simple diagnostic tests. Therefore, we hypothesize that RPA is amenable to broad application for molecular diagnostics while offering fast turnaround time (<1 hour).

2.3 Project objectives

To answer adequately our hypothesis, we investigated three elements of RPA technology. The first element was to explore the specificity of the RPA mechanism since little is known about the tolerance of RPA to sequence mismatches more particularly in closely-related non-target sequences. This will help in establishing guidelines to develop adapted RPA diagnostic assays. The second element was to evaluate RPA on clinical samples since at the time there was no demonstration of RPA directly on clinical materials. Finally, we investigated the possibility of integrating RPA into an automated lab-on-chip system that can offer a rapid sample-to-answer. Three objectives introduce the subsequent chapters of this thesis as follows:

1) - Establishment of primer/probe design guidelines required for a specific RPA diagnostic test. This objective forms chapter III of this thesis and constitutes a post-print version of a published article in *Molecular and Cellular Probes.*

2) - Evaluation of the clinical performance of RPA compared to rtPCR for the rapid detection of *Streptococcus agalactiae*. This objective forms chapter IV of this thesis and constitutes a post-print version of a published article in *Clinical Chemistry*.

3) - Integration of RPA in an automated microfluidic centripetal system called "blade" for the rapid detection of *Streptococcus agalactiae*. This objective forms chapter V of this thesis and constitutes a manuscript in progress.

Chapter

III. Influence of sequence mismatches on the specificity of recombinase polymerase amplification technology

Published *in Molecular and Cellular Probes, April 2015; Volume 29; Issue 2; Pages 116-121.*

Foreword

Student contribution in the preparation of the article:

The role of the student is reflected mainly in the development of the research project and the experimental protocol. That is carrying out experiments and interpretation of results. Manuscript submission along with tracking editor/reviewers decisions were also carried out by the student.

Co-authors contribution in the preparation of the article:

Michel G. Bergeron, Gale Stewart and Maurice Boissinot supervised the research project. Gale Stewart and Maurice Boissinot validated results interpretation. Dominique Boudreau provided the sequences of bacterial species used in this project. All co-authors have also contributed in the revision and annotation of the manuscript.

Information on the status of the article:

The article under the subject of "*Influence of sequence mismatches on the specificity of recombinase polymerase amplification technology*" is published in the journal of *Molecular and Cellular Probes*, April 2015 [Online: http://www.ncbi.nlm.nih.gov/pubmed/25481659]. Chapter III of this thesis comprises the post-print version of the article which is licensed under a Creative Commons Attribution-Non Commercial-No Derivatives 4.0 International License (CC-BY-NC-ND).

Influence of sequence mismatches on the specificity of recombinase polymerase amplification technology

Author names and affiliations:

Rana K. Daher[1, 2], Gale Stewart[1], Maurice Boissinot[1], Dominique K. Boudreau[1] and Michel G. Bergeron[1]

[1] Centre de recherche du CHU de Québec, Centre de recherche en infectiologie de l'Université Laval (CRI) _CHUL, 2705, Boul. Laurier - RC 709, Québec, Québec, G1V 4G2, Canada

[2] Département de microbiologie-immunologie, faculté de médecine, Université Laval, 1045 Avenue de la Médecine, Québec, Québec, G1V 0A6, Canada

Corresponding author:

Dr Michel G. Bergeron

Centre de recherche en infectiologie de l'Université Laval (CRI) _CHUL,

2705, Boul. Laurier - RC 709, Québec, Québec, G1V 4G2, Canada

Telephone: 418-525-4444, ext. 48753

Fax: 418-654-2197

E-mail: michel.g.bergeron@crchul.ulaval.ca

Résumé

Tout en étant spécifique, la technologie *recombinase polymerase amplification* (RPA) permet l'amplification des acides nucléiques à une seule température. Cette dernière dépend de trois protéines clés soient des recombinases, des protéines de liaison de l'ADN simple brin et des polymérases. La performance de la RPA par rapport aux séquences mésappariées entre espèces étroitement apparentées n'est pas bien documentée. De plus, l'influence du nombre et la distribution des mésappariements sur la réaction d'amplification RPA n'a pas été démontré. Nous avons étudié la spécificité de la RPA en testant des espèces étroitement apparentées qui portent dans leur séquence des mésappariements d'origines naturelles pour: 1) la séquence du gène *tuf* de *Pseudomonas aeruginosa* et/ou de *Mycobacterium tuberculosis* et 2) la séquence du gène *cfb* de *Streptococcus agalactiae*. De même, pour évaluer l'impact du nombre et la distribution des mésappariements, 14 types d'amorces mutées sont générées synthétiquement pour détecter cinq espèces bactériennes de haute pertinence diagnostique soient *Clostridium difficile*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* ainsi que *Bacillus atrophaeus* pour laquelle nous utilisons les spores comme contrôle interne dans des essais diagnostiques. Un total de 87 amorces mésappariées ont été testées lors de cette étude. Nous avons observé que des mésappariements (n>1) à l'extrémité 3' des amorces spécifiques entravaient la réaction de la RPA. En outre, trois mésappariements couvrant les deux extrémités (5'-3') et le centre de la séquence de l'amorce affectaient négativement le rendement de la RPA. Nous avons démontré également que la spécificité de la RPA est multifactorielle. Par conséquent, son application dans des contextes cliniques doit être sélectionné et validé *a priori*. Nous recommandons que la sélection d'un gène cible doit tenir compte de la présence de gènes non-cibles étroitement liés. Il est alors conseillé de choisir des régions cibles avec un nombre élevé de mésappariements (≥36%, par rapport à la longueur de l'amplicon) par rapport aux espèces étroitement liées et le meilleur choix sera pour un gène cible unique.

Abstract

Recombinase polymerase amplification (RPA) technology relies on three major proteins, recombinase proteins, single-strand binding proteins, and polymerases to specifically amplify nucleic acid sequences in an isothermal format. The performance of RPA with respect to sequence mismatches of closely-related non-target molecules is not well documented and the influence of the number and distribution of mismatches in DNA sequences on RPA amplification reaction is not well understood. We investigated the specificity of RPA by testing closely-related species bearing naturally occurring mismatches for the *tuf* gene sequence of *Pseudomonas aeruginosa* and/or *Mycobacterium tuberculosis* and for the *cfb* gene sequence of *Streptococcus agalactiae*. In addition, the impact of the number and distribution of mismatches on RPA efficiency was assessed by synthetically generating 14 types of mismatched forward primers for detecting five bacterial species of high diagnostic relevance such as *Clostridium difficile*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis* as well as *Bacillus atrophaeus* subsp. *globigii* for which we use the spores as internal control in diagnostic assays. A total of 87 mismatched primers were tested in this study. We observed that target specific RPA primers with mismatches (n>1) at their 3'extremity hampered RPA reaction. In addition, 3 mismatches covering both extremities and the center of the primer sequence negatively affected RPA yield. We demonstrated that the specificity of RPA was multifactorial. Therefore its application in clinical settings must be selected and validated *a priori*. We recommend that the selection of a target gene must consider the presence of closely-related non-target genes. It is advisable to choose target regions with a high number of mismatches (≥36%, relative to the size of amplicon) with respect to closely-related species and the best case scenario would be by choosing a unique target gene.

Keywords

RPA; RecA proteins; homology search; sequence mismatches; specificity

List of abbreviations

RPA, recombinase polymerase amplification

PCR, polymerase chain reaction

HIV, human immunodeficiency virus

tuf, elongation factor Tu

cfb, CAMP-factor B

3.1 Introduction

Since its initial publication in 2006, the isothermal recombinase polymerase amplification (RPA) technology for nucleic acids detection has been applied in various fields such as: agriculture, veterinary, and human medicine [1-6]. RPA has even been integrated into microfluidic devices [7-13]. However, there is no extensive study on the influence of base pair mismatches in DNA sequence on RPA amplification reaction. With PCR, the specificity of amplification resides mainly in primer design and the annealing temperature [14]. Failure to discriminate between closely-related target molecules results in false positives thus biasing the diagnosis and lowering the treatment efficacy [15]. In addition for RPA, the specificity is dependent upon the recombinase enzymes function during the homology search [16]. The manufacturer of RPA reagents, TwistDx, provides general guidelines for designing RPA primers. These include avoiding long tracks of one particular nucleotide or a large number of small repeats. High or low GC content (>70% or <30%, respectively) must also be avoided. RPA primers should be 30 to 35 bases long, longer primers (>45 bases) are not recommended since they increase the likelihood of secondary-structures [17]. In RPA technology, the primer-target homology search is induced by the formation of a nucleoprotein filament made of recombinases/ssDNA (*i.e.* primers and/or probes) that invades dsDNA and initiates strand-exchange thus allowing the elongation of the annealed primer/probe resulting in exponential amplification of the DNA target. This mechanism is well described by Piepenburg *et al.* in their first publication of the RPA technology [16]. Little is known about how recombinase enzymes recognize homology [18]. The performance of RPA with respect to sequence mismatches of closely-related molecules is not well documented. In addition, sequence mismatches (unspecific byproducts) are most likely to occur for RPA since it functions at low temperatures (37° – 42°C) [12]. Boyle *et al.* has demonstrated that RPA is capable of detecting multiple HIV-1 subtypes tolerating up to 9 mismatches across the primer/probe regions [19]. Another study showed that the performance of RPA assay for the detection of foot-and-mouth disease virus RNA was not affected by 5 mismatches within RPA primers and exo-probe [20]. The purpose of

this study is to provide insight into RPA performance with respect to sequence variants of target and non-target molecules to facilitate RPA assay design and application. Specifically, the actual study addresses two basic questions which are: 1) Can RPA technology specifically discriminate sequence mismatches among closely-related species? 2) What is the impact of the number and distribution of mismatches on RPA amplification efficiency? To answer these questions, we first investigated the specificity of RPA reaction by testing closely-related species showing sequence mismatches either to highly conserved gene or to unique gene. By definition, highly conserved gene are present in all bacteria with a certain level of variability between species while unique genes are specific to a bacterial species, with sometimes distant homologous and highly variable sequences found in other species [21]. We chose *tuf* (elongation factor Tu) of *Pseudomonas aeruginosa* and of *Mycobacterium tuberculosis* as a conserved gene. For the unique gene, we chose *cfb* (CAMP-factor B) of *Streptococcus agalactiae*. Furthermore, the impact of the number and distribution of mismatches on RPA reaction efficiency was assessed by synthetically generating 14 types of mismatched primers by introducing nucleotide changes into the forward primer sequence for detecting five bacterial species of high diagnostic relevance and another sporulating bacterium useful as an internal control in diagnostic assays [22].

3.2 Materials and Methods

3.2.1 Primers design

Wild type forward and reverse primers for each of the strains listed in table 3.2 were designed according to the protocol of TwistAmp® Basic kit (TwistDx, UK). Primers varied in length between 30 - 33 bases (table 3.1). Mismatched primers were designed by modifying 1 to 3 bases at the extremities (5'-3') or the center of the corresponding forward primer of assays 1^{α} to 6^{α} (table 3.2). Twelve types of

mismatches ((i) to (xii)) were introduced into the forward primer sequence of assays 1^{α} to 5^{α}. Mismatch type (xiii) was generated for assays 1^{α}, 3^{α}, and 4^{α} while mismatch type (xiv) was generated for assays 2^{α} and 5^{α}. Forward primer of assay 6^{α} was subjected to mismatch types (i), (ii), (iii), (v), (vi), and (vii) (figure 3.1). The modifications were mainly transitions (replacing adenine (A) by guanine (G) and cytosine (C) by thymine (T), and vice-versa). The choice for transitions was guided by the work of Newton *et al.* who demonstrated that purine/purine or pyrimidine/pyrimidine mismatches were more destabilizing for PCR amplification than purine/pyrimidine mismatches [23]. In the case of types (i) and (v) mismatches for certain species, the modification was by changing one nucleotide type into all 3 remaining possibilities. See supplemental data tables 3.6 to 3.11 for complete sequences of mismatched primers.

3.2.2 RPA reaction conditions

RPA reaction for each sample was done in duplicate in all assays. A 50 µl total volume RPA mixture was made of 2.4 µl forward primer (either wild type or mismatched), 2.4 µl reverse primer, 29.5 µl rehydration buffer, and 11.2 µl nuclease-free water. One freeze-dried reagent pellet included in the TwistAmp® basic kit was added to every reaction tube and vortexed. Then, 2 µl of genomic DNA of the bacterial species used in this study (table 3.2) were added separately at 1000 genome copies per reaction. The reaction mixture was set on a cold block to start a synchronized amplification in all the reaction tubes. The amplification reaction was initiated by the addition of 2.5 µl of magnesium acetate solution (14 mM) to each reaction. The reaction tubes were vortexed briefly and then incubated in a PTC-200 Thermo Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) at 39 °C for 20 min. Reaction tubes were removed from the instrument and vortexed briefly after the first 4 min of incubation then reinserted to complete the incubation for 16 min. RPA amplicons were purified with QIAquick PCR Purification Kit (QIAGEN Inc., Toronto, ON, Canada) according to the manufacturer's protocol. Purified amplicons were visualized using 2% agarose gel electrophoresis with 0.25 µg/ml of

ethidium bromide in Tris-borate-EDTA buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3). Amplicon sizes were verified with a 1-kb molecular weight DNA ladder (Invitrogen, Carlsbad, CA, USA). The intensity of specific amplicon band was quantified according to the manual instructions of AlphaImager™ 2000 (Alpha Innotech Corporation, San Leandro, CA, USA). The intensity of the amplicon band generated with wild type primers was considered as baseline and served for comparison of amplicon yields with mismatched primers. Bands higher than 250 ng were represented by (+++), (++) for those between 150 – 250 ng, (+) for those between 50 – 150 ng, (±) for those less than 50 ng ,which were also considered as inconclusive results, and 0 when bands were absent.

3.2.3 Bacterial species used

Experimentation was performed with purified genomic DNA from 22 bacterial species of high diagnostic relevance (table 3.2). A total of 14 bacterial species of human *in vitro* diagnostics interest were used for RPA primers specificity assays 1 & 2 targeting the highly conserved *tuf* gene. Six closely-related *Mycobacterium* species and eight bacterial species harboring variable mismatches to primer sequences served for the analysis of *M. tuberculosis* or *P. aeruginosa* primers specificity, respectively (table 3.2). Five *Streptococcus* species bearing distantly-related *cfb* homologous genes were used in specificity assay 3 of *S. agalactiae* primers targeting the unique *cfb* gene (table 3.2). In addition, six bacterial species served for the analysis on the effect of forward primers mismatches distribution on the specificity of amplification (assays 1^{α} to 6^{α}) which were *P. aeruginosa, M. tuberculosis*, *S. agalactiae*, *B. atrophaeus* subsp. *globigii*, *C. difficile*, and *S. aureus* (table 3.2). Strains were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and the Collection du Centre de Recherche en Infectiologie (CCRI) (Québec City, Canada). Bacterial DNA was isolated according to the manufacturer's protocol of BioSprint 15 DNA blood kit (Qiagen, Mississauga, Ontario, Canada) using a KingFisher™ mL Magnetic Particle Processors (Thermo Fisher Scientific, Waltham, MA, USA). Alternatively, a GNOME® DNA kit

(Qbiogene Inc., Carlsbad, CA, USA) was used. Genomic DNA of each of the strains was used at 1000 genome copies per reaction.

3.3 Results

3.3.1 Evaluation of RPA basic wild type primers

All six wild type primer pair sets of table 3.1 were evaluated for the specific amplification of their corresponding target and all primer sets were shown to be specific. All six primer pair sets generated a specific band on a 2% agarose gel electrophoresis (data not shown). The intensity of the RPA product band generated with the wild type primers was considered as baseline and served for comparison of amplicon yields with mismatched primers used in assays 1^{α} to 6^{α}.

3.3.2 Specificity analysis of highly conserved and unique target genes

For assay 1, 7 bacterial species bearing variable sequence mismatches with *P. aeruginosa tuf* gene primer pair regions were tested for amplification with RPA TwistAmp® basic kit by using wild type forward and reverse primer TPaerF and TPaerR, respectively in the reaction solution. The number of mismatches varied from 5 to 23 with respect to the sequence of the latter primers used in this study (figure 3.2). Their distribution with respect to TPaerF and TPaerR was randomly scattered along the 5'-3' ends and the internal region of the forward and/or reverse primer (Supplemental table 3.3). According to the phylogenetic representation in figure 3.2 [24], we observed that in most cases the number of mismatches increased as the bacterial species receded from the position of reference species herein *P. aeruginosa*. In addition, as the number of mismatches increased RPA amplification ceased. We observed that from 8 mismatches total on both forward and reverse primers (*e.g. E. hoshinae*) the RPA reaction was negatively affected

and as the mismatch increased up to 23 (*e.g. G. sanguinis*) the inhibition of RPA amplification was still noticeable (figure 3.2).

For assay 2, 5 *Mycobacterium* species bearing variable sequence mismatches in *M. tuberculosis tuf* gene primer pair regions were tested for amplification with RPA by using wild type forward and reverse primer TMtubF and TMtubR, respectively in the reaction solution. The number of mismatches varied from 3 to 7 with respect to the sequence of the latter primers (figure 3.3). Their distribution with respect to TMtubF was mainly a single mismatch at the 12th base upstream the 5'end or an additional mismatch near or at the 3'end of the primer. With respect to TMtubR, mismatches were randomly scattered along 5'-3' ends and the internal region of the reverse primer (Supplemental table 3.4). According to the phylogenetic representation in figure 3.3 [24], we observed that 6 and 7 mismatches negatively affected the RPA reaction. Taking for example *M. gordonae* and *M. smegmatis* who had same number of mismatches (n=6), amplification of the first species with TMtubF and TMtubR primer pair was reduced whereas it was absent for *M. smegmatis* (figure 3.3).

For assay 3, 4 *Streptococcus* species bearing variable sequence mismatches in *S. agalactiae cfb* gene primer pair regions were tested for amplification with RPA by using wild type forward and reverse primer cfbSagF and cfbSagR, respectively in the reaction solution. The number of mismatches varied from 22 to 33 with respect to the sequences of the latter primers (figure 3.4). Their distribution with respect to cfbSagF and cfbSagR was randomly scattered from extremities (5'-3') to the internal region of the forward and reverse primer (Supplemental table 3.5). RPA amplification reaction was only observed in *S. agalactiae* but not in other tested *Streptococcus* species, probably due to high number of mismatches (figure 3.4).

3.3 Effect of mismatches distribution on RPA amplification reaction

Fourteen mismatch variations were synthesized to generate mismatched forward primers for each of *P. aeruginosa*, *M. tuberculosis*, *S. agalactiae*, *B. atrophaeus*, *C.*

difficile, and *S. aureus* (Supplemental tables 3.6 to 3.11). The number of mismatches varied from 1 to 3 and the distribution covered the 5' end, the center, as well as the 3' end of the forward primer sequence (figure 3.1). The effect of mismatches distribution varied between the strains reflecting the fact that the primer sequence for each strain had different GC content (ranging from 30.3% to 69.7%) (table 3.1). A single mismatch at the 3' end of forward primer (type (i)) had a slight effect on RPA amplification of *P. aeruginosa*, *B. atrophaeus*, and *C. difficile* but not of *M. tuberculosis*, *S. agalactiae*, and *S. aureus*. Changing base type in these strains aborted RPA amplification in most of cases (Supplemental tables 3.6 – 3.8 & 3.10 – 3.11). Two mismatches at the 3' end, either type (ii) or (iii) reduced the efficiency of RPA amplification especially when they were adjacent. This effect was seen for most of the strains. On the other hand, we observed that when 2 adjacent mismatches were 4 bases away from 3' end of the primer (type (iv)), the amplification with RPA was slightly affected. One or two mismatches at or near the 5' end (types (v, vi & vii)) of the forward primer had minor effect on the efficiency of amplification in most of the strains except for *S. agalactiae* and *S. aureus* (Supplemental tables 3.8 & 3.11). For most of the strains, RPA amplification was mildly affected by one or two mismatches at the center (types (viii & ix)). When mismatches were 4 bases away from each extremity of the primer (type (x)), only *M. tuberculosis* and *C. difficile* gave reduced RPA yield compared to their baseline wild-type primer. With one mismatch at either 5' or 3' end with an additional mismatch at the center (types (xi & xii)), the effect was variable among strains. For example, the amplification was inhibited for *S. agalactiae* but reduced for *M. tuberculosis* (Supplemental tables 3.7 & 3.8). With 3 mismatches covering both extremities (5' & 3') and the center of the primer (types (xiii) & (xiv)), the amplification of *P. aeruginosa*, *M. tuberculosis*, and *S. agalactiae* with the mismatched primer was negatively affected and even suppressed in the case of *S. agalactiae* (Supplemental tables 3.6, 3.7 & 3.8).

3.4 Discussion

In the present study, we demonstrated that the specificity of RPA technology is dependent on the number and distribution of mismatches in the sequence of molecules closely-related to the target gene sequence. We noticed that non-specific RPA results were mostly generated for species bearing mismatches in a highly conserved housekeeping gene sequence as it is the case for *tuf* gene. False positives were obtained from non-target species bearing ~ 7 mismatches (11%) in the primer pair regions of 66 bases of either *P. aeruginosa* or *M. tuberculosis*. Boyle *et al.* have obtained comparable results where RPA tolerated up to 9 mismatches (8%) across the primer pair and probe regions having a total of 118 bases [19]. In another study, Patil *et al.* showed that at 12% mismatches across the incoming ssDNA (83 bases), the rate of strand-exchange was reduced by 50% [18]. As strand-exchange rate decreases due to sequence mismatches, RPA yield decreases as well. Our results showed that the discrimination of mismatches was more successful in the case of *Streptococcus* species bearing distantly-related *cfb* homologous gene with a minimum of 22 mismatches (36%) across the target primer pair sequences of 63 bases in total. As a whole, this indicates that the discrimination of closely-related molecules with RPA technology could be enhanced by choosing target regions highly polymorphic (≥36%, relative to the size of amplicon) to non-target regions.

As in PCR, the distribution of mismatches had an impact on RPA amplification efficiency. We observed that mismatches at the 3'end of the primer sequence reduced the efficiency of RPA amplification in most of cases. With PCR, this phenomenon is explained by the inability of the polymerase enzyme to bind to the mismatched 3' end, there for failing to initiate elongation. Consequently, PCR primers only tolerates minimal mismatch within the last 5-6 bases of the 3' end [25]. Several studies have been published to understand how recombinases recognize DNA sequences with high affinity and catalyze subsequent homologous pairing and strand-exchange [18, 26-29]. Morel *et al.* have demonstrated that the size and position of mismatched insertions in either ssDNA or dsDNA affected

strand-exchange. Large mismatched insertions (150 - 200 bp) at 5'end of dsDNA were more tolerated than a 22 bp mismatched insertion at its 3' end [26]. This could be explained in part by the fact that RecA proteins promoted unidirectional strand-exchange from 5' to 3' end with respect to ssDNA at 3 - 10 bp/s [30]. It correlates with our results since in most of cases RPA amplification reaction was less affected with mismatches either at 5' end or at internal primer region than 3' end mismatches. Zhang *et al.* showed that ATP-hydrolysis assisted RecA proteins through homology recognition and appropriate strand-exchange [29], 20 - 30/min per RecA monomer [30]. Upon ATP-hydrolysis and during homology search, RecA nucleoprotein filament was able to recognize 18 (23%) mismatched bases causing structural perturbations in an 80-mer DNA sequence (*e.g.* G-T mismatches) but not those without base perturbations (*e.g.* inosine (I)-C mismatches) [29]. Knowing that there is 1 RecA monomer per 3 nucleotides [30], RecA strand-exchange, hence RPA yield, is most probably influenced by the number and the relative distribution of mismatches causing base-stacking perturbations with respect to the size of the target DNA sequence. Taking all together, this might explain RPA amplification failure that was observed in our study for certain forward primers mismatched sequences. However, it is also likely that part of what we observed in the RPA system is not only due to the action of RecA but also partly the result of the action of the polymerase Sau used in the RPA kit. It is well known that strand extension by polymerases is hampered by mismatches at the 3' extremity of primers.

In the present study, we were not able to get predictable results for the effect of single mismatch on RPA reaction efficiency. Results varied between different target sequences which had variable GC content as low as 30.3% (*e.g.* TCdifF) and up to 69.7% (*e.g.* TMtubF). The variability of results between the six RPA assays (1^{α} to 6^{α}) that served for this study (despite their respective mismatched forward primers having the same number and distribution of mismatches) could be explained in part by the difference in the GC content of the target sequences. We noticed a general tendency that mismatched sequences having medium to high GC content (*e.g. P. aeruginosa* at 63.6% GC and *B. atrophaeus* at 48.4% GC, respectively) had less impact on RPA reaction efficiency than those with low or

very high GC content (*e.g. C. difficile* at 30.3% GC, *S. aureus* at 33.3% GC, *S. agalactiae* at 39.4% GC, and *M. tuberculosis* at 69.7% GC, respectively). This could be explained in part by a work of Patil *et al.* who showed that the rate of *Escherichia coli* RecA protein strand-exchange was directly impacted by the % GC content such as at 70% GC, the rate was slower than at 50% and 60% GC content [18]. Thus, with slow rate of strand-exchange due to low or very high GC content sequences, RPA yield will probably be reduced.

We demonstrated that the specificity of RPA was multifactorial therefore its application in clinical settings must be selected and validated *a priori*. In addition to the recommendations of the manufacturer regarding RPA primer design and selection [17, 31], our results suggest that mismatched bases should be avoided at the 3' end of either forward or reverse primers. Moreover, avoiding mismatched bases that cover the extremities and the center of the oligonucleotide sequence is encouraged since it had also deleterious effect on RPA yield. We recommend that the selection of target molecule must consider the presence of closely-related non-target molecules. For optimal specificity, it is advisable to choose target regions with high number of mismatches. The best case scenario would be by choosing a unique target gene as in the case of *S. agalactiae cfb* gene. The discrimination of single base mismatches was not predictable in our study design. However, one possible advantage of RPA is its tolerance to a certain number of mismatches. An interesting application would be for diagnostics where emerging variants also need to be detected but not necessarily discriminated from the wild-type target. On the other hand, Shin *et al.* demonstrated that RPA can be applied for the screening of DNA sequence alterations in human diseases. They developed an isothermal solid-phase RPA protocol capable of detecting single-nucleotide variation in bladder cancer cells genomic DNA by adding mutant-primer (mismatched) directly into the RPA reaction solution [7]. Ultimately, further investigation is needed to assess the performance of RPA detection systems either in gel, real-time or lateral flow formats towards the discrimination of sequence variations in closely-related targets. It is known that with both exo or nfo probes, RPA probes will also serve as forward primer after their initial cleavage by nuclease enzyme [16]. This could

ultimately bias the specificity analysis especially when attempting to discriminate sequence mismatches in closely related targets.

In conclusion, we were able to provide insight about the specificity of RPA technology and how the degree of mismatch in closely-related molecules impacted RPA reaction specificity.

Acknowledgments

This work was supported by the program "Projets d'innovation en technologies de la santé" and funded by Fonds de recherche du Québec - Santé (FRQS) and Fonds de recherche du Québec – Nature et technologies (FRQNT) – GenePOC. We thank, Jean-Luc Simard for providing us with useful technical help to accomplish this work.

References

[1] Ahmed A, van der Linden H, Hartskeerl RA. Development of a recombinase polymerase amplification assay for the detection of pathogenic *Leptospira*. Int J Environ Res Public Health. 2014;11:4953-64.

[2] Daher RK, Stewart G, Boissinot M, Bergeron MG. Isothermal recombinase polymerase amplification assay applied to the detection of group B streptococci in vaginal/anal samples. Clin Chem. 2014;60:660-6.

[3] Hill-Cawthorne GA, Hudson LO, El Ghany MF, Piepenburg O, Nair M, Dodgson A, et al. Recombinations in staphylococcal cassette chromosome *mec* elements compromise the molecular detection of methicillin resistance in *Staphylococcus aureus*. PLoS ONE. 2014;9:e101419.

[4] Mekuria TA, Zhang S, Eastwell KC. Rapid and sensitive detection of Little cherry virus 2 using isothermal reverse transcription-recombinase polymerase amplification. J Virol Methods. 2014;205C:24-30.

[5] Sakai K, Trabasso P, Moretti ML, Mikami Y, Kamei K, Gonoi T. Identification of fungal pathogens by visible microarray system in combination with isothermal gene amplification. Mycopathologia. 2014;178:11-26.

[6] Euler M, Wang Y, Heidenreich D, Patel P, Strohmeier O, Hakenberg S, et al. Development of a panel of recombinase polymerase amplification assays for the detection of biothreat agents. J Clin Microbiol. 2013;51:1110-7.

[7] Shin Y, Perera AP, Kim KW, Park MK. Real-time, label-free isothermal solid-phase amplification/detection (ISAD) device for rapid detection of genetic alteration in cancers. Lab Chip. 2013;13:2106-14.

[8] Escadafal C, Faye O, Sall AA, Weidmann M, Strohmeier O, von Stetten F, et al. Rapid molecular assays for the detection of yellow fever virus in low-resource settings. PLoS Negl Trop Dis. 2014;8:e2730.

[9] Kim TH, Park J, Kim CJ, Cho YK. Fully Integrated Lab-on-a-Disc for Nucleic Acid Analysis of Food-Borne Pathogens. Anal Chem. 2014;86:3841-48.

[10] Lutz S, Weber P, Focke M, Faltin B, Hoffmann J, Muller C, et al. Microfluidic lab-on-a-foil for nucleic acid analysis based on isothermal recombinase polymerase amplification (RPA). Lab Chip. 2010;10:887-93.

[11] Erh-Chia Yeh, Lee LP. One-step digital plasma separation for molecular diagnostics. 17th International Conference on Miniaturized Systems for Chemistry and Life Sciences. Freiburg, Germany: μTAS; 2013. p. 1323-25.

[12] Kersting S, Rausch V, Bier F, von Nickisch-Rosenegk M. Multiplex isothermal solid-phase recombinase polymerase amplification for the specific and fast DNA-based detection of three bacterial pathogens. Microchim Acta. 2014:1-9.

[13] Rohrman BA, Richards-Kortum RR. A paper and plastic device for performing recombinase polymerase amplification of HIV DNA. Lab Chip. 2012;12:3082-8.

[14] Bej AK, Mahbubani MH, Atlas RM. Amplification of nucleic acids by polymerase chain reaction (PCR) and other methods and their applications. Crit Rev Biochem Mol Biol. 1991;26:301-34.

[15] Boyle B, Dallaire N, MacKay J. Evaluation of the impact of single nucleotide polymorphisms and primer mismatches on quantitative PCR. BMC Biotechnol. 2009;9:75.

[16] Piepenburg O, Williams CH, Stemple DL, Armes NA. DNA detection using recombination proteins. PLoS Biol. 2006;4:1115-21.

[17] TwistDx. Instruction Manuals.

[18] Patil KN, Singh P, Muniyappa K. DNA binding, coprotease, and strand exchange activities of mycobacterial RecA proteins: Implications for functional diversity among RecA nucleoprotein filaments. Biochemistry. 2011;50:300-11.

[19] Boyle DS, Lehman DA, Lillis L, Peterson D, Singhal M, Armes N, et al. Rapid detection of HIV-1 proviral DNA for early infant diagnosis using recombinase polymerase amplification. MBio. 2013;4.

[20] Abd El Wahed A, El-Deeb A, El-Tholoth M, Abd El Kader H, Ahmed A, Hassan S, et al. A portable reverse transcription recombinase polymerase amplification assay for rapid detection of foot-and-mouth disease virus. PLoS ONE. 2013;8:e71642.

[21] Petti CA. Detection and identification of microorganisms by gene amplification and sequencing. Clin Infect Dis. 2007;44:1108-14.

[22] Picard FJ, Gagnon M, Bernier MR, Parham NJ, Bastien M, Boissinot M, et al. Internal control for nucleic acid testing based on the use of purified *Bacillus atrophaeus* subsp. *globigii* spores. J Clin Microbiol. 2009;47:751-7.

[23] Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, et al. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res. 1989;17:2503-16.

[24] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013;30:2725-9.

[25] Álvarez-Fernández R. Chapter One - Explanatory Chapter: PCR Primer Design. In: Jon L, editor. Methods Enzymol: Academic Press; 2013. p. 1-21.

[26] Morel P, Stasiak A, Ehrlich SD, Cassuto E. Effect of length and location of heterologous sequences on RecA-mediated strand exchange. J Biol Chem. 1994;269:19830-5.

[27] Savir Y, Tlusty T. RecA-Mediated Homology Search as a Nearly Optimal Signal Detection System. Mol Cell. 2010;40:388-96.

[28] Chen Z, Yang H, Pavletich NP. Mechanism of homologous recombination from the RecA-ssDNA/dsDNA structures. Nature. 2008;453:489-4.

[29] Zhang Z, Yoon D, LaPorte JR, Chen J. Appropriate initiation of the strand exchange reaction promoted by RecA protein requires ATP hydrolysis. J Mol Biol. 2001;309:29-43.

[30] Jain SK, Cox MM, Inman RB. On the role of ATP hydrolysis in RecA protein-mediated DNA strand exchange. III. Unidirectional branch migration and extensive hybrid DNA formation. J Biol Chem. 1994;269:20653-61.

[31] Boyle DS, McNerney R, Teng Low H, Leader BT, Perez-Osorio AC, Meyer JC, et al. Rapid detection of *Mycobacterium tuberculosis* by recombinase polymerase amplification. PLoS ONE. 2014;9:e103091.

Table 3.1. Wild type primer pair sequences used in this study

Assay	Species	Gene	Primer ID*	Sequence (5'- 3')	GC%	Length (b)	Amplicon size (bp)	Sequence accession number
1	*P. aeruginosa*	*tuf*	TPaerF	CTGGACTCCTACATTCCGGAGCCGGTTCGTGCC	63.6	33	227	AE004091
			TPaerR	AATGTTCCGCAAGCTGCTCGACGAAGGTCGTGC	57.6	33		
2	*M. tuberculosis*	*tuf*	TMtubF	CAGATGGACGGTGCGATCCTGGTGGTCGCCGCC	69.7	33	234	AL123456
			TMtubR	GAGCGCCGAGACCCGCACAACCGGGGCGTCCTC	75.8	33		
3	*S. agalactiae*	*cfb*	cfbSagF	TTTCACCAGCTGTATTAGAAGTACATGCTGATC	39.4	33	235	AE014283
			cfbSagR	ACTGTCTCAGGGTTGGCACGCAATGAAGTC	53.3	30		
4	*B. atrophaeus*	*atpD*	ABglF	AGAGGTCGCACTTCATTTAGGCGACGATACT	48.4	31	227	CP007640
			ABglR	ACGGAGCTTGTCTGTGAATCGGATCTTTCTC	48.4	31		
5	*C. difficile*	*tuf*	TCdifF	ATGGTAGATGATGAAGAGTTATTAGAGTTAGTA	30.3	33	234	AM180355
			TCdifR	CTCTACTGGCATTAAGAATGGTTTATCTGTATC	36.4	33		
6	*S. aureus*	*mec*	mecAF	TTCATATGACGTCTATCCATTTATGTATGGCAT	33.3	33	234	NC_007793
			mecAR	TTGTAACCACCCCAAGATTTATCTTTTTGCCAA	36.4	33		

* Forward primers: names ending with "F"; Reverse primers: names ending with "R".

Table 3.2. List of bacterial species used in this study

Assay*	Species	Strain ID	Application
1	*Pseudomonas aeruginosa*	CCRI-701	Specificity of detection (*tuf* gene of *P. aeruginosa*)
	Pseudomonas oryzihabitans	ATCC 43272	
	Aeromonas caviae	CCRI-14843	
	Edwardsiella hoshiniae	ATCC 33379	
	Salmonella enterica subsp. *arizonae*	ATCC 13314	
	Salmonella enterica subsp. *enterica* Gallinarum	ATCC 9184	
	Mobiluncus curtisii subsp. *holmesii*	ATCC 35242	
	Globicatella sanguinis	ATCC 51173	
2	*Mycobacterium tuberculosis*	ATCC 25177	Specificity of detection (*tuf* gene of *M. tuberculosis*)
	Mycobacterium avium	ATCC 25291	
	Mycobacterium gordonae	CCRI-5433	
	Mycobacterium smegmatis	CCRI-5439	
	Mycobacterium senegalense	CCRI-19276	
	Mycobacterium septicum	CCRI-19275	
3	*Streptococcus agalactiae*	ATCC 12973	Specificity of detection (*cfb* gene of *S. agalactiae*)
	Streptococcus uberis	ATCC 19436	
	Streptococcus canis	ATCC 43496	
	Streptococcus pyogenes	ATCC 19615	
	Streptococcus iniae	CCRI-16688	
4	*Bacillus atrophaeus* subsp. *globigii*	CCRI-9827	Specific detection of *atpD* gene of *B. globigii*
5	*Clostridium difficile*	ATCC 9689	Specific detection of *tuf* gene of *C. difficile*
6	*Staphylococcus aureus*	CCRI-9583	Specific detection of *mec* gene
1 α	*Pseudomonas aeruginosa*	CCRI-701	Effect of mismatches distribution
2 α	*Mycobacterium tuberculosis*	ATCC 25177	
3 α	*Streptococcus agalactiae*	ATCC 12973	
4 α	*Bacillus atrophaeus* subsp. *globigii*	CCRI-9827	
5 α	*Clostridium difficile*	ATCC 9689	
6 α	*Staphylococcus aureus*	CCRI-9583	

* Assays 1 to 6 were made using only wild type primers listed in table 3.1. Assays 1α to 6 α were made with wild-type and mismatched primers according to figure 3.1.

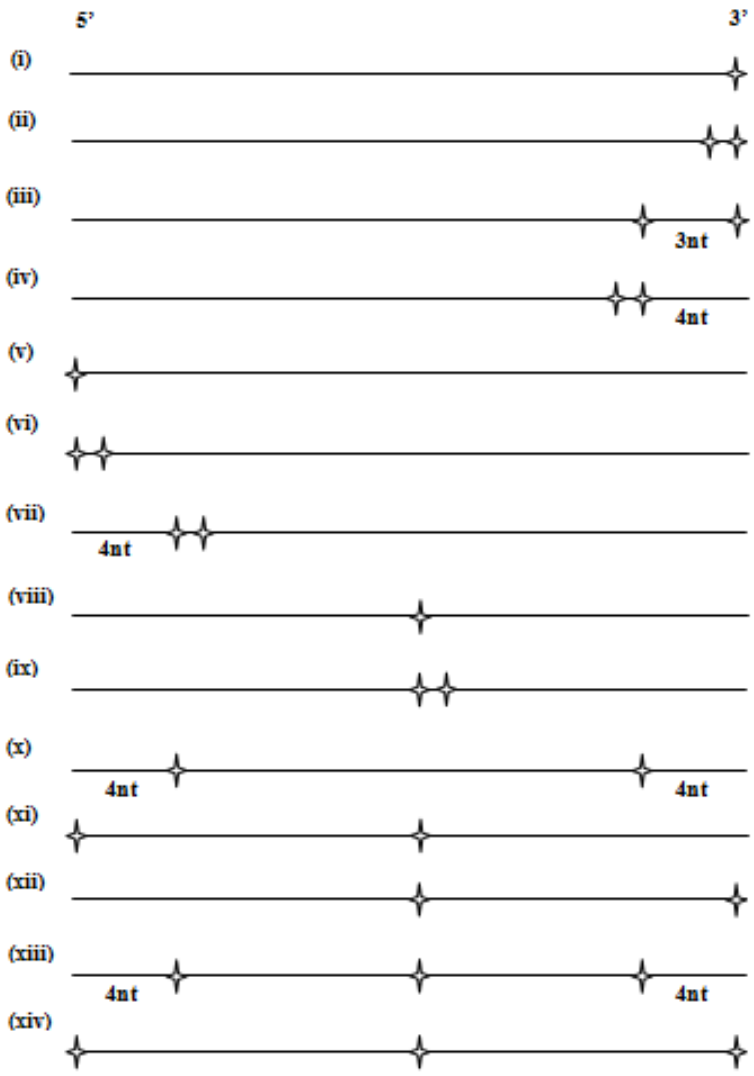

Figure 3.1. Schematic representation of relative position of mismatches introduced into the forward primer sequence of assays 1^{α} to 6^{α}

Dark line represents the sequence of the primer and stars represent the actual position of the mismatch. Roman numbers (i) to (xiv) represent the different types of mismatches made upon the primer.

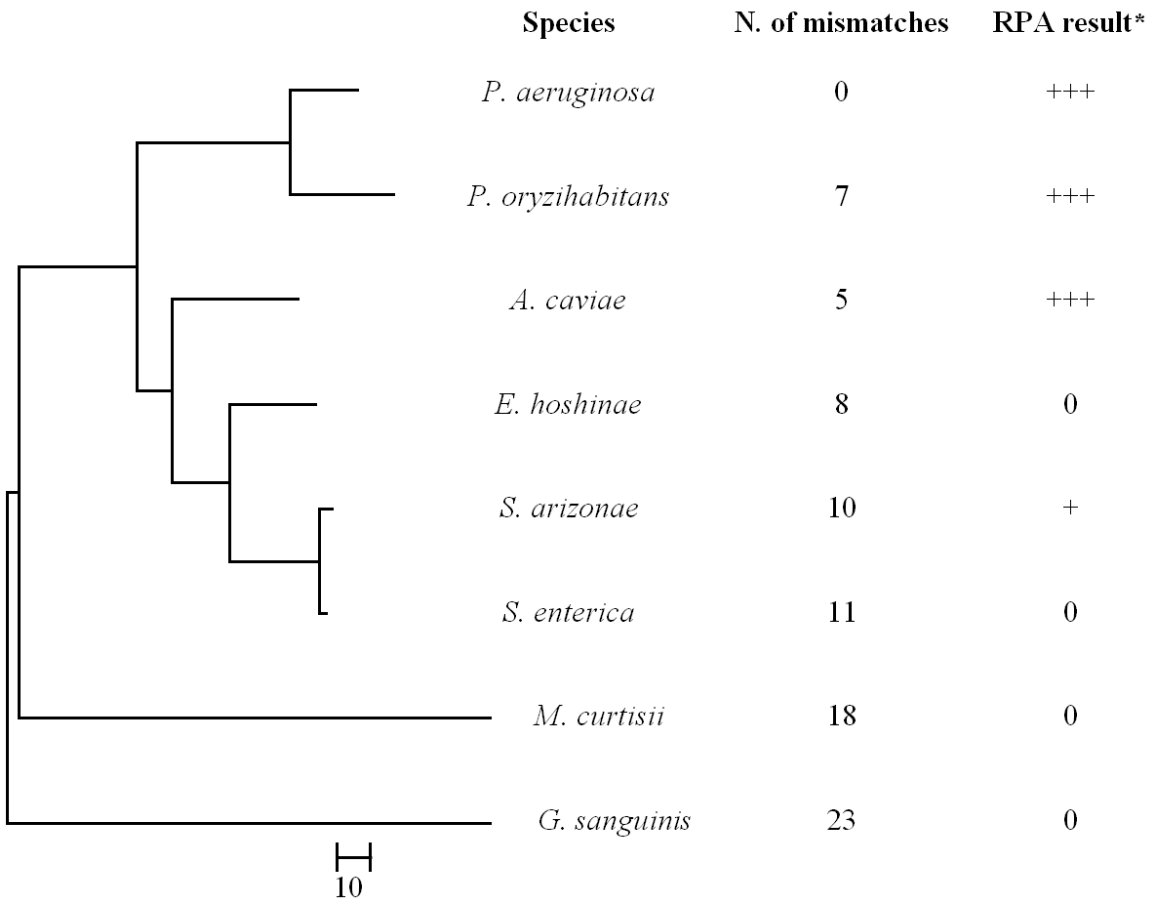

Figure 3.2. Phylogenetic representation of 8 bacterial species bearing variable sequence mismatches in *tuf* gene primer pair sequences of *P. aeruginosa*

Concatenate sequences regions corresponding to forward and reverse primers were aligned using MEGA version 6.05 [24]. The dendrogram was constructed with the neighbor-joining method using the number of mismatches model of the MEGA software. The scale bar indicates the number of mismatches: as the seven bacterial species recedes from the reference *P. aeruginosa*, the number of mismatches found in the primer pair sequence increases in most cases. * Intensity of the band on 2% agarose gel corresponding to RPA amplicons. (+++), > 250 ng; (+), 50 - 150 ng; 0, absence of band.

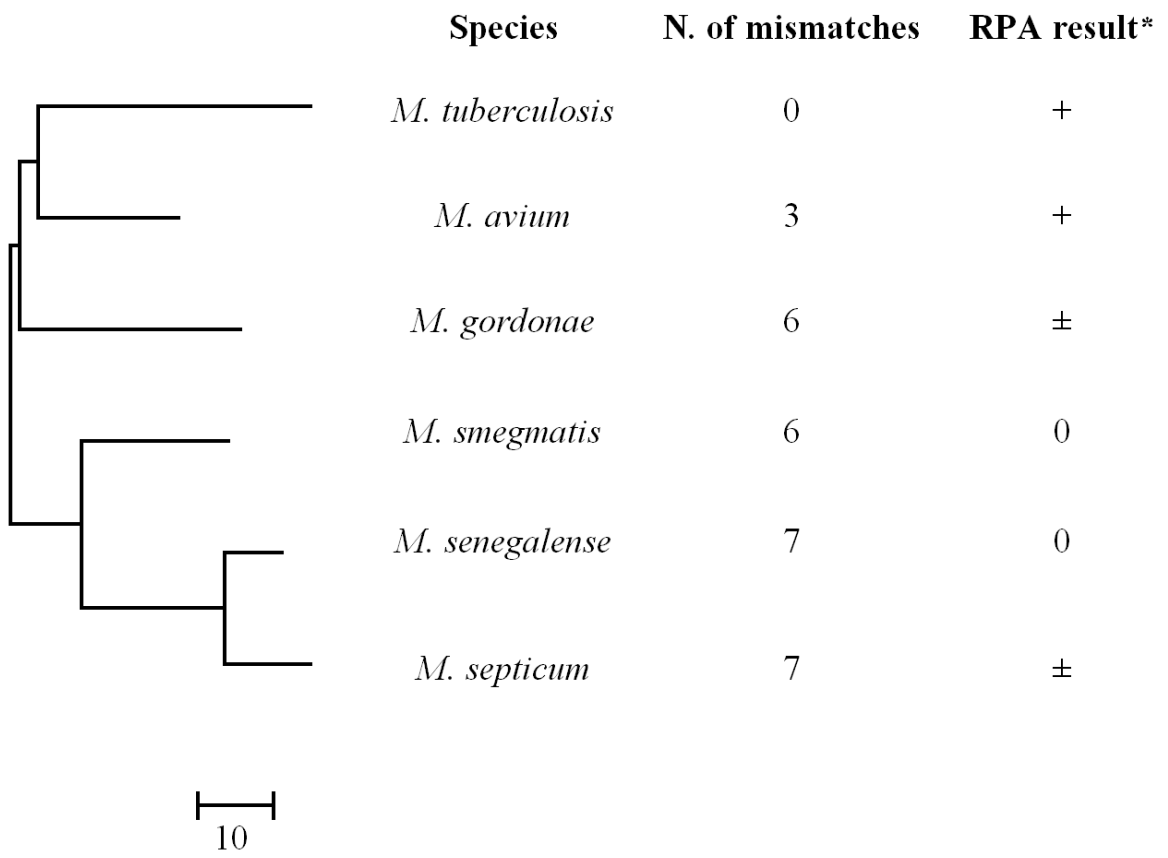

Figure 3.3. Phylogenetic representation of 6 *Mycobacterium* species bearing variable sequence mismatches in *tuf* gene primer pair sequences of *M. tuberculosis*

Concatenate sequences regions corresponding to forward and reverse primers were aligned using MEGA version 6.05 [24]. The dendrogram was constructed with the neighbor-joining method using the number of mismatches model of the MEGA software. The scale bar indicates the number of mismatches: as the five *Mycobacterium* species recedes from the reference *M. tuberculosis*, the number of mismatches found in the primer pair sequence increases. * Intensity of the band on 2% agarose gel corresponding to RPA amplicons. (+), 50 - 150 ng; (±), < 50 ng; 0, absence of band.

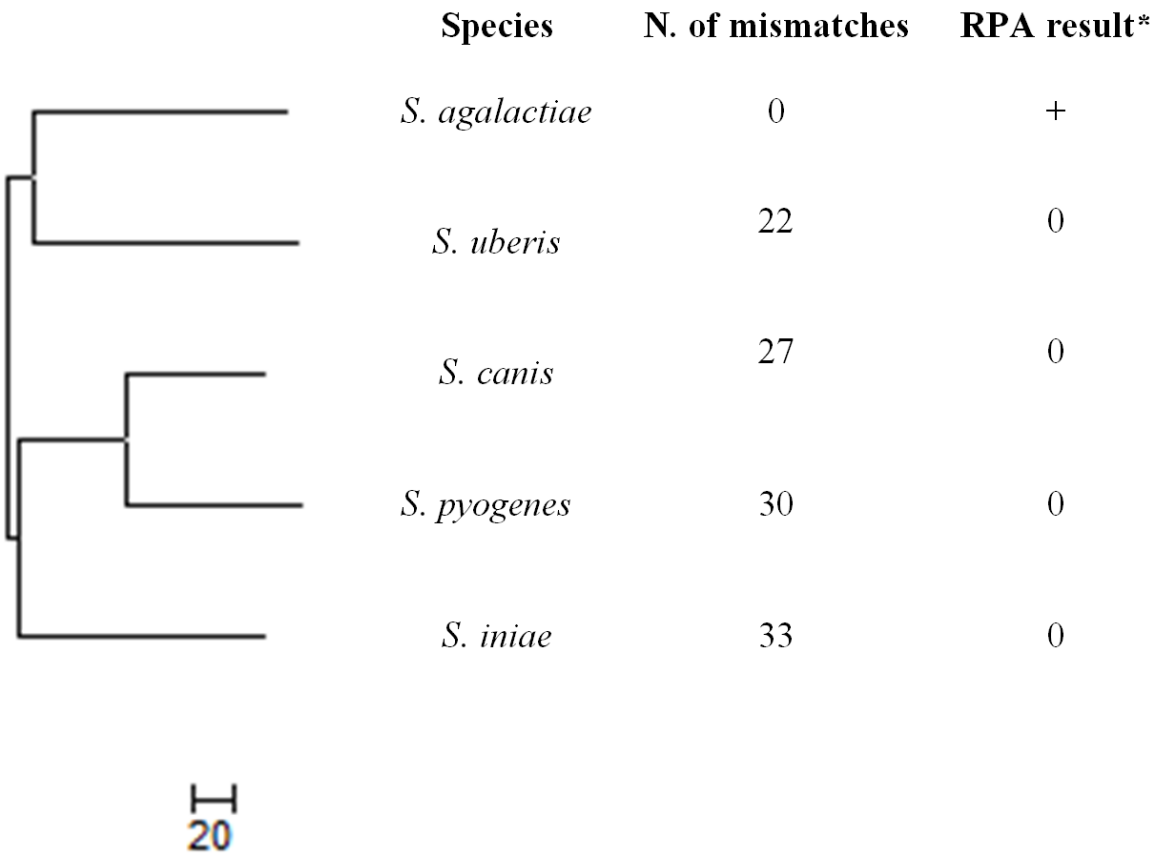

Figure 3.4. Phylogenetic representation of 5 *Streptococcus* species bearing variable sequence mismatches in *cfb* gene primer pair sequences of *S. agalactiae*

Concatenate sequences regions corresponding to forward and reverse primers were aligned using MEGA version 6.05 [24]. The dendrogram was constructed with the neighbor-joining method using the number of mismatches model of the MEGA software. The scale bar indicates the number of mismatches: as the four streptococcal species recedes from the reference *S. agalactiae*, the number of mismatches found in the primer pair sequence increases. * Intensity of the band on 2% agarose gel corresponding to RPA amplicons. (+), 50 - 150 ng; 0, absence of band.

Supplemental data tables

Table 3.3. Mismatches distribution for *P. aeruginosa tuf* gene sequence

Assay	Species	Forward primer sequence* (5' – 3')	Reverse primer sequence (5' -3')	N. of mismatches	RPA result**
1	*P. aeruginosa*	CTGGACTCCTACATTCCGGAGCCGGTTCGTGCC	TTACAAGGCGTTCGACGAGCTGCTTCCAGCACG	0	+++
	P. oryzihabitans	CTGGACTCCTACATCCCCGAGCCCGAGCGTGCC	TTACAAGGCGTTCGACGACCTGCTTCCGGCACG	7	+++
	A. caviae	CTGGACACCTACATTCCGGAGCCGGAGCGTGCC	TTACAAGGCGTTTGACGACCTGCTTCCAGCACG	5	+++
	E. hoshinae	CTGGACTCCTACATTCCGGAACCTGAGCGTGAC	TTACAAGGCGTTTGACGACCTGCTTCCGGCACG	8	0
	S. arizonae	CTGGACTCTTACATCCCGGAACCAGAGCGTGCG	TTACAAGGCGTTTGACGACCTGCTTCCGGCACG	10	+
	S. enterica	CTGGATTCTTACATCCCGGAACCAGAGCGTGCG	TTACAAGGCGTTTGACGACCTGCTTCCGGCACG	11	0
	M. curtisii	GTCGATACCTACATTCCTGAGCCTGTTCGTGAC	TTACAAGGTGTTCAGGTACCTGCTTCGGATGCG	18	0
	G. sanguinis	GTAGATACATACATTCCAGAACCAGTTCGTGAT	TTACAAGGCATTTAATAATCTAATGCGACTTCG	23	0

* Black box shows the relative distribution of mismatch; ** Intensity of the band on 2% agarose gel corresponding to RPA amplicons. (+++), > 250 ng; (+), 50 - 150 ng; 0, absence of band.

Table 3.4. Mismatches distribution for *M. tuberculosis tuf* gene sequence

Assay	Species	Forward primer sequence* (5' – 3')	Reverse primer sequence (5' -3')	N. of mismatches	RPA result**
2	*M. tuberculosis*	CAGATGGACGGTGCGATCCTGGTGGTCGCCGCC	GAGCGCCGAGACCCGCACAACCGGGGCGTCCTC	0	+
	M. avium	-AGATGGACGGGGCGATCCTGGTGGTCGCCGCC	GAGCGCCGACACCCGCACCACCGGGGCGTCCTC	3	+
	M. gordonae	-AGATGGACGGGGCGATCCTGGTGGTCGCCGCC	CAGCGCCGAGACCCGCACCACCGGAGCTTCCTC	6	±
	M. smegmatis	-AGATGGACGGCGCGATCCTGGTGGTCGCCGCC	GAGCGCCGAGACCCGCACCACGGGGGCCTCCTC	6	0
	M. senegalense	-AGATGGACGGCGCGATCCTGGTGGTCGCCGCC	CAGCGCGGAGACCCGCATCACCGGGGCGTCCTC	7	0
	M. septicum	-AGATGGACGGCGCGATCCTGGTGGTCGCCGCG	CAGCGCGGAGACCCGCATAACCGGGGCATCCTC	7	±

* Black box shows the relative distribution of mismatch; (-) unknown base due to incomplete sequence; ** Intensity of the band on 2% agarose gel corresponding to RPA amplicons. (+), 50 - 150 ng; (±), < 50 ng; 0, absence of band.

Table 3.5. Mismatches distribution for *S. agalactiae cfb* gene sequence

Assay	Species	Forward primer sequence* (5' – 3')	Reverse primer sequence (5' -3')	N. of mismatches	RPA result**
3	*S. agalactiae*	TTTCACCAGCTGTATTAGAAGTACATGCTGATC	GACTTCATTGCGTGCCAACCCTGAGACAGT	0	+
	S. uberis	TTTCCCCAATTTTAACAAGTGTCCAAGCAAATC	AACTTCACTTAGAGCTAATCCTGAAACAAT	22	0
	S. canis	TTCCAACCGTAGGTCAGTCAGTTTTTGCTGATA	AACATCTGTTCGTTTCAGCCCTGATACCAT	27	0
	S. pyogenes	TTTCAACTATGACGCACTCAGTTTTAGCTGATG	GAGTTCTATACGCTTCAATACTGAAACGAT	30	0
	S. iniae	TGTCTTTAGCTGCGCCATCTGTTTTAGCTGATG	AAGCTCTCTTAAATTTAATCCAGAAACTAT	33	0

* Black box shows the relative distribution of mismatch; ** Intensity of the band on 2% agarose gel corresponding to RPA amplicons. (+), 50 - 150 ng; 0, absence of band.

Table 3.6. Sequence of mismatched primers of *P. aeruginosa tuf* gene

Assay	Strain	Gene	Primer ID	Sequence* (5' – 3')	RPA result**
1[α]	*P. aeruginosa*	*tuf*	TPaerF	CTGGACTCCTACATTCCGGAGCCGGTTCGTGCC	++
			TPaerF1	CTGGACTCCTACATTCCGGAGCCGGTTCGTGCT	+
			TPaerF2	CTGGACTCCTACATTCCGGAGCCGGTTCGTGCA	0
			TPaerF3	CTGGACTCCTACATTCCGGAGCCGGTTCGTGCG	++
			TPaerF4	CTGGACTCCTACATTCCGGAGCCGGTTCGTGTT	0
			TPaerF5	CTGGACTCCTACATTCCGGAGCCGGTTCATGCT	±
			TPaerF6	CTGGACTCCTACATTCCGGAGCCGGTTTATGCC	++
			TPaerF7	TTGGACTCCTACATTCCGGAGCCGGTTCGTGCC	++
			TPaerF8	ATGGACTCCTACATTCCGGAGCCGGTTCGTGCC	++
			TPaerF9	GTGGACTCCTACATTCCGGAGCCGGTTCGTGCC	++
			TPaerF10	TCGGACTCCTACATTCCGGAGCCGGTTCGTGCC	+++
			TPaerF11	CTGGGTTCCTACATTCCGGAGCCGGTTCGTGCC	++
			TPaerF12	CTGGACTCCTACATTCTGGAGCCGGTTCGTGCC	+++
			TPaerF13	CTGGACTCCTACATTTTGGAGCCGGTTCGTGCC	++
			TPaerF14	CTGGGCTCCTACATTCCGGAGCCGGTTCATGCC	+++
			TPaerF15	TTGGACTCCTACATTCTGGAGCCGGTTCGTGCC	+++
			TPaerF16	CTGGACTCCTACATTCTGGAGCCGGTTCGTGCT	±
			TPaerF17	CTGGGCTCCTACATTCTGGAGCCGGTTCATGCC	+

* black box shows the position and the type of mismatch; ** Intensity of the band on 2% agarose gel corresponding to RPA amplicons. (+++), > 250 ng; (++), 150 – 250 ng; (+), 50 - 150 ng; (±), < 50 ng; 0, absence of band.

Table 3.7. Sequence of mismatched primers of *M. tuberculosis tuf* gene

Assay	Strain	Gene	Primer ID	Sequence* (5' – 3')	RPA result**
2[α]	*M. tuberculosis*	*tuf*	TMtubF	CAGATGGACGGTGCGATCCTGGTGGTCGCCGCC	++
			TMtubF1	CAGATGGACGGTGCGATCCTGGTGGTCGCCGCT	0
			TMtubF2	CAGATGGACGGTGCGATCCTGGTGGTCGCCGCA	0
			TMtubF3	CAGATGGACGGTGCGATCCTGGTGGTCGCCGCG	0
			TMtubF4	CAGATGGACGGTGCGATCCTGGTGGTCGCCGTT	±
			TMtubF5	CAGATGGACGGTGCGATCCTGGTGGTCGTCGCT	0
			TMtubF6	CAGATGGACGGTGCGATCCTGGTGGTCATCGCC	±
			TMtubF7	TAGATGGACGGTGCGATCCTGGTGGTCGCCGCC	+
			TMtubF8	TGGATGGACGGTGCGATCCTGGTGGTCGCCGCC	++
			TMtubF9	CAGACAGACGGTGCGATCCTGGTGGTCGCCGCC	+
			TMtubF10	CAGATGGACGGTGCGACCCTGGTGGTCGCCGCC	++
			TMtubF11	CAGATGGACGGTGCGGCCCTGGTGGTCGCCGCC	+
			TMtubF12	CAGACGGACGGTGCGATCCTGGTGGTCGTCGCC	+
			TMtubF13	TAGATGGACGGTGCGACCCTGGTGGTCGCCGCC	+
			TMtubF14	CAGATGGACGGTGCGACCCTGGTGGTCGCCGCT	±
			TMtubF15	TAGATGGACGGTGCGACCCTGGTGGTCGCCGCT	±

* black box shows the position and the type of mismatch; ** Intensity of the band on 2% agarose gel corresponding to RPA amplicons. (++), 150 – 250 ng; (+), 50 - 150 ng; (±), < 50 ng; 0, absence of band.

Table 3.8. Sequence of mismatched primers of *S. agalactiae cfb* gene

Assay	Strain	Gene	Primer ID	Sequence* (5' – 3')	RPA result**
3[α]	*S. agalactiae*	*cfb*	cfbSagF	TTTCACCAGCTGTATTAGAAGTACATGCTGATC	+
			cfbSagF1	TTTCACCAGCTGTATTAGAAGTACATGCTGATT	+
			cfbSagF2	TTTCACCAGCTGTATTAGAAGTACATGCTGATA	0
			cfbSagF3	TTTCACCAGCTGTATTAGAAGTACATGCTGATG	0
			cfbSagF4	TTTCACCAGCTGTATTAGAAGTACATGCTGACT	0
			cfbSagF5	TTTCACCAGCTGTATTAGAAGTACATGCCGATT	±
			cfbSagF6	TTTCACCAGCTGTATTAGAAGTACATGTCGATC	++
			cfbSagF7	CTTCACCAGCTGTATTAGAAGTACATGCTGATC	0
			cfbSagF8	GTTCACCAGCTGTATTAGAAGTACATGCTGATC	±
			cfbSagF9	ATTCACCAGCTGTATTAGAAGTACATGCTGATC	+
			cfbSagF10	CCTCACCAGCTGTATTAGAAGTACATGCTGATC	0
			cfbSagF11	TTTCGTCAGCTGTATTAGAAGTACATGCTGATC	0
			cfbSagF12	TTTCACCAGCTGTATTAAAAGTACATGCTGATC	±
			cfbSagF13	TTTCACCAGCTGTATTAAGAGTACATGCTGATC	+
			cfbSagF14	TTTCGCCAGCTGTATTAGAAGTACATGCCGATC	+
			cfbSagF15	CTTCACCAGCTGTATTAAAAGTACATGCTGATC	0
			cfbSagF16	TTTCACCAGCTGTATTAAAAGTACATGCTGATT	0
			cfbSagF17	TTTCGCCAGCTGTATTAAAAGTACATGCCGATC	0

* Black box shows the position and the type of mismatch; ** Intensity of the band on 2% agarose gel corresponding to RPA amplicons. (++), 150 – 250 ng; (+), 50 - 150 ng; (±), < 50 ng; 0, absence of band.

Table 3.9. Sequence of mismatched primers of *B. atrophaeus atpD* gene

Assay	Strain	Gene	Primer ID	Sequence* (5' – 3')	RPA result**
4[α]	*B. atrophaeus*	*atpD*	ABglF	AGAGGTCGCACTTCATTTAGGCGACGATACT	++
			ABglF1	AGAGGTCGCACTTCATTTAGGCGACGATACC	++
			ABglF2	AGAGGTCGCACTTCATTTAGGCGACGATATC	±
			ABglF3	AGAGGTCGCACTTCATTTAGGCGACGGTACC	+
			ABglF4	AGAGGTCGCACTTCATTTAGGCGACAGTACT	+
			ABglF5	GGAGGTCGCACTTCATTTAGGCGACGATACT	+++
			ABglF6	GAAGGTCGCACTTCATTTAGGCGACGATACT	+++
			ABglF7	AGAGACCGCACTTCATTTAGGCGACGATACT	+++
			ABglF8	AGAGGTCGCACTTCACTTAGGCGACGATACT	+++
			ABglF9	AGAGGTCGCACTTCACCTAGGCGACGATACT	+++
			ABglF10	AGAGATCGCACTTCATTTAGGCGACGGTACT	++
			ABglF11	GGAGGTCGCACTTCACTTAGGCGACGATACT	+++
			ABglF12	AGAGGTCGCACTTCACTTAGGCGACGATACC	+++
			ABglF13	AGAGATCGCACTTCACTTAGGCGACGGTACT	+++

* black box shows the position and the type of mismatch; ** Intensity of the band on 2% agarose gel corresponding to RPA amplicons. (+++), > 250 ng; (++), 150 – 250 ng; (+), 50 - 150 ng; (±), < 50 ng.

Table 3.10. Sequence of mismatched primers of *C. difficile tuf* gene

Assay	Strain	Gene	Primer ID	Sequence* (5' – 3')	RPA result**
5[α]	*C. difficile*	*tuf*	TCdifF	ATGGTAGATGATGAAGAGTTATTAGAGTTAGTA	+
			TCdifF1	ATGGTAGATGATGAAGAGTTATTAGAGTTAGTG	+
			TCdifF2	ATGGTAGATGATGAAGAGTTATTAGAGTTAGTC	++
			TCdifF3	ATGGTAGATGATGAAGAGTTATTAGAGTTAGTT	+
			TCdifF4	ATGGTAGATGATGAAGAGTTATTAGAGTTAGCG	0
			TCdifF5	ATGGTAGATGATGAAGAGTTATTAGAGTCAGTG	0
			TCdifF6	ATGGTAGATGATGAAGAGTTATTAGAGCCAGTA	0
			TCdifF7	GTGGTAGATGATGAAGAGTTATTAGAGTTAGTA	+
			TCdifF8	GCGGTAGATGATGAAGAGTTATTAGAGTTAGTA	+
			TCdifF9	ATGGCGGATGATGAAGAGTTATTAGAGTTAGTA	+
			TCdifF10	ATGGTAGATGATGAAGGGTTATTAGAGTTAGTA	+
			TCdifF11	ATGGTAGATGATGAAAGGTTATTAGAGTTAGTA	+
			TCdifF12	ATGGCAGATGATGAAGAGTTATTAGAGTCAGTA	±
			TCdifF13	GTGGTAGATGATGAAGGGTTATTAGAGTTAGTA	+++
			TCdifF14	ATGGTAGATGATGAAGGGTTATTAGAGTTAGTG	++
			TCdifF15	GTGGTAGATGATGAAGGGTTATTAGAGTTAGTG	+

* Black box shows the position and the type of mismatch; ** Intensity of the band on 2% agarose gel corresponding to RPA amplicons. (+++), > 250 ng; (++), 150 – 250 ng; (+), 50 - 150 ng; (±), < 50 ng; 0, absence of band.

Table 3.11. Sequence of mismatched primers of *S. aureus mec* gene

Assay	Strain	Gene	Primer ID	Sequence* (5' – 3')	RPA result**
6[α]	*S. aureus*	*mec*	mecAF	TTCATATGACGTCTATCCATTTATGTATGGCAT	+
			mecAF1	TTCATATGACGTCTATCCATTTATGTATGGCAA	0
			mecAF2	TTCATATGACGTCTATCCATTTATGTATGGCAC	+
			mecAF3	TTCATATGACGTCTATCCATTTATGTATGGCAG	0
			mecAF4	TTCATATGACGTCTATCCATTTATGTATGGCGC	0
			mecAF5	TTCATATGACGTCTATCCATTTATGTATAGCAC	0
			mecAF6	ATCATATGACGTCTATCCATTTATGTATGGCAT	0
			mecAF7	CTCATATGACGTCTATCCATTTATGTATGGCAT	0
			mecAF8	GTCATATGACGTCTATCCATTTATGTATGGCAT	0
			mecAF9	CCCATATGACGTCTATCCATTTATGTATGGCAT	0
			mecAF10	TTCACGTGACGTCTATCCATTTATGTATGGCAT	0

* Black box shows the position and the type of mismatch; ** Intensity of the band on 2% agarose gel corresponding to RPA amplicons. (+), 50 - 150 ng; 0, absence of band.

Supplemental data figures

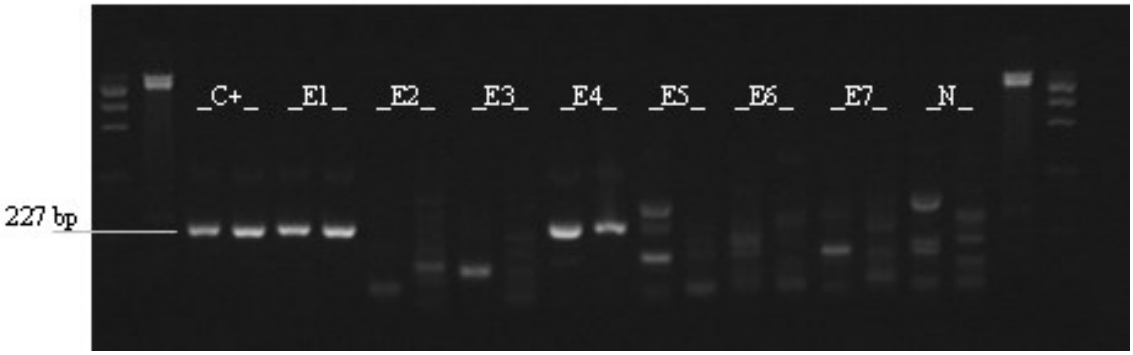

Figure 3.5. RPA-gel electrophoresis results of mismatches distribution for *P. aeruginosa tuf* gene sequence

Expected amplicon size of 227 bp verified with a 1-kb molecular weight DNA ladder. C+ = *P. aeruginosa;* E1 = *A. caviae;* E2 = *E. hoshinae;* E3 = *M. curtisii;* E4 = *P. oryzihabitans* ; E5 = *S. arizonae;* E6 = *S. enterica;* E7 = *G. sanguinis;* N = Non-template control.

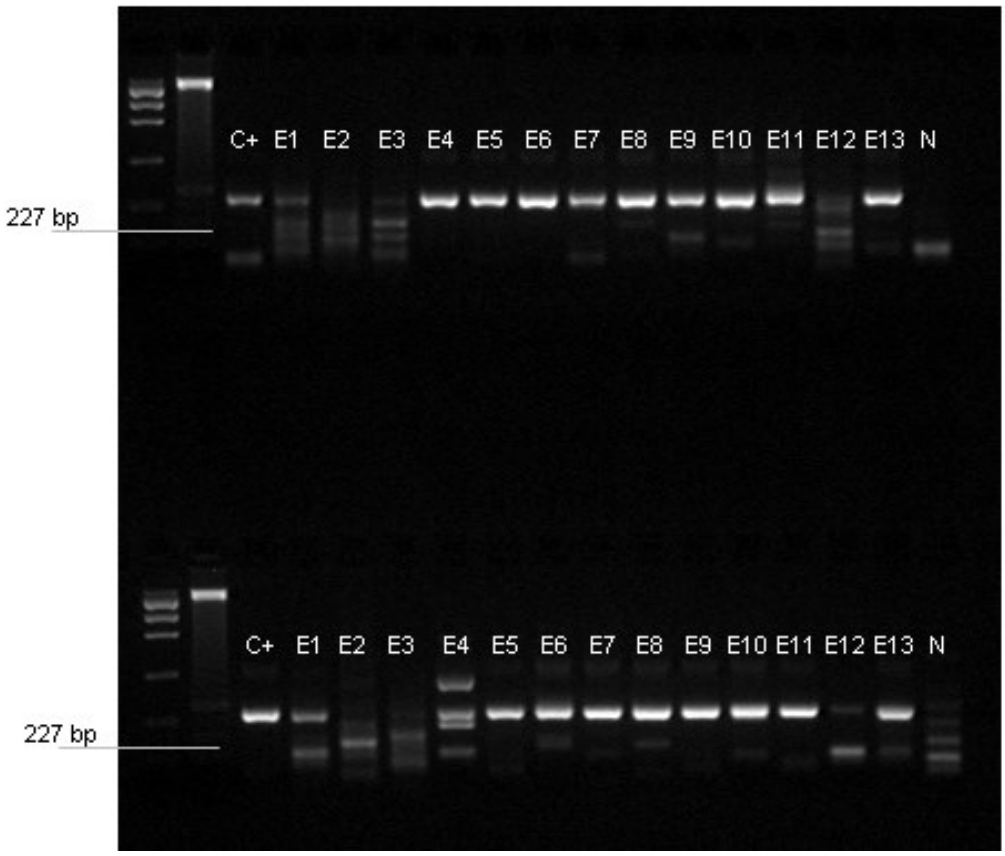

Figure 3.6. RPA-gel electrophoresis results of the specificity analysis with synthetic mismatched forward primers for *P. aeruginosa tuf* gene sequence

Expected amplicon size of 227 bp verified with a 1-kb molecular weight DNA ladder. C+ = TPaerF/TPaerR wild-type primers; E1 = TPaerF1/TPaerR; E2 = TPaerF4/TPaerR; E3 = TPaerF5/TPaerR; E4 = TPaerF6/TPaerR; E5 = TPaerF7/TPaerR; E6 = TPaerF10/TPaerR; E7 = TPaerF11/TPaerR; E8 = TPaerF12/TPaerR; E9 = TPaerF13/TPaerR; E10 = TPaerF14/TPaerR; E11 = TPaerF15/TPaerR; E12 = TPaerF16/TPaerR; E13 = TPaerF17/TPaerR; N = Non-template control. TPaerF1, TPaerF4-7 and TPaerF10-17 correspond to mismatched forward primers for *P. aeruginosa tuf* gene sequence as shown in table 3.6.

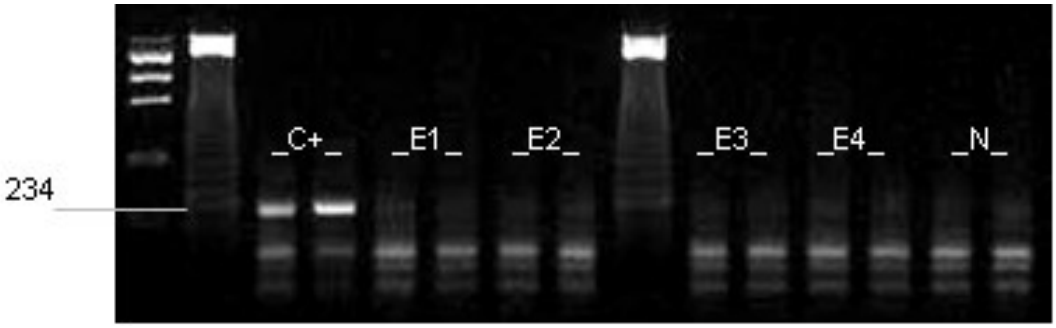

Figure 3.7. RPA-gel electrophoresis results of mismatches distribution for *S. agalactiae cfb* gene sequence

Expected amplicon size of 234 bp verified with a 1-kb molecular weight DNA ladder. C+ = *S. agalactiae;* E1 = *S. canis;* E2 = *S. iniae;* E3 = *S. pyogenes;* E4 = *S. uberis;* N = Non-template control.

Chapter

IV. Isothermal recombinase polymerase amplification assay applied to the detection of group B streptococci in vaginal/anal samples

Published *in Clinical Chemistry, April 2014; Volume 60; Issue 4; Pages 660-666.*

Foreword

Student contribution in the preparation of the article:

The role of the student is reflected mainly in the development of the research project and the experimental protocol. That is carrying out experiments and interpretation of results. Manuscript submission along with tracking editor/reviewers decisions were also carried out by the student.

Co-authors contribution in the preparation of the article:

Michel G. Bergeron, Gale Stewart and Maurice Boissinot supervised the research project. Gale Stewart and Maurice Boissinot validated the interpretation of results. All co-authors have also contributed to the revision and annotation of the manuscript.

Information on the status of the article:

The article under the subject of "*Isothermal recombinase polymerase amplification assay applied to the detection of group B streptococci in vaginal/anal samples*" has been published in the journal of *Clinical Chemistry*, April 2014 [Online: http://www.ncbi.nlm.nih.gov/pubmed/24463560]. Chapter IV of this thesis comprises the post-print version of this article which is reproduced with permission from the American Association for Clinical Chemistry.

Isothermal recombinase polymerase amplification assay applied to the detection of group B streptococci in vaginal/anal samples

Running title:

Clinical performance of RPA

Author names and Affiliations:

Rana K. Daher[1, 2], Gale Stewart[1], Maurice Boissinot[1] and Michel G. Bergeron[1]

[1] Centre de recherche du CHU de Québec, Centre de recherche en infectiologie de l'Université Laval (CRI) _CHUL, 2705, Boul. Laurier - RC 709, Québec, Québec, G1V 4G2, Canada

[2] Département de microbiologie-immunologie, faculté de médecine, Université Laval, 1045 Avenue de la Médecine, Québec, Québec, G1V 0A6, Canada

Corresponding author:

Dr Michel G. Bergeron

Centre de recherche en infectiologie de l'Université Laval (CRI) _CHUL,

2705, Boul. Laurier - RC 709, Québec, Québec, G1V 4G2, Canada

Telephone: 418-525-4444, ext. 48753

Fax: 418-654-2197

E-mail: michel.g.bergeron@crchul.ulaval.ca

Résumé

Contexte: Les infections à streptocoques du groupe B sont la principale cause de la septicémie et la méningite chez les nouveau-nés. Un test rapide et fiable pour la détection de ce pathogène au moment de l'accouchement est nécessaire pour le traitement précoce des nouveau-nés. Les techniques d'amplification isotherme telles que la *recombinase polymerase amplification* (RPA) ont des avantages par rapport à la PCR en termes de la vitesse de réaction et la simplicité opérationnelle.

Méthodes: Nous avons étudié la performance clinique de la RPA pour le diagnostic des streptocoques du groupe B dans des échantillons vaginaux/anaux provenant de 50 femmes enceintes. Nous avons également comparé la limite de détection et la spécificité analytique de cette technique isotherme avec la PCR en temps réel (rtPCR).

Résultats: Par rapport à la rtPCR, l'amplification isotherme avec la RPA a démontré une sensibilité clinique de 96% et une spécificité clinique de 100%. La limite de détection fut de 98 copies génome avec 100% de spécificité analytique pour 15 souches bactériennes et/ou fongiques présentes naturellement dans la flore vaginale/anale. Le temps d'obtention des résultats avec la RPA est en moins de 20 minutes comparé à 45 minutes pour l'essai rtPCR; un échantillon positif fut détecté en 8 minutes.

Conclusions: Nous démontrons le potentiel de la technologie d'amplification isotherme RPA comme une méthode efficace pour le diagnostic moléculaire clinique tout en étant simple et plus rapide que la PCR/rtPCR. La RPA offre un grand potentiel pour le diagnostic des acides nucléiques au chevet du patient.

Abstract

Background: Group B streptococcal infections are the leading cause of sepsis and meningitis in newborns. A rapid and a reliable method for the detection of this pathogen at the time of delivery is needed for the early treatment of neonates. Isothermal amplification techniques such as recombinase polymerase amplification have advantages relative to PCR in terms of the speed of reaction and simplicity.

Methods: We studied the clinical performance of recombinase polymerase amplification for the screening of group B streptococci in vaginal/anal samples from 50 pregnant women. We also compared the limit of detection and the analytical specificity of this isothermal assay to real-time PCR (rtPCR).

Results: Compared to rtPCR, the recombinase polymerase amplification assay showed a clinical sensitivity of 96% and a clinical specificity of 100%. The limit of detection was 98 genome copies and the analytical specificity was 100% for a panel of 15 bacterial and/or fungal strains naturally found in the vaginal/anal flora. Time-to-result for the recombinase polymerase amplification assay was less than 20 minutes compared to 45 minutes for the rtPCR assay; a positive sample could be detected as early as 8 minutes.

Conclusions: We demonstrate the potential of isothermal recombinase polymerase amplification assay as a clinically useful molecular diagnostic tool that is simple and faster than PCR/rtPCR. Recombinase polymerase amplification offers great potential for nucleic acid based diagnostics at the point-of-care.

Keywords

GBS, isothermal amplification, real-time PCR, Point-of-care

List of abbreviations

GBS, group B streptococcus

CDC, centers for disease control and prevention

rtPCR, real-time PCR

LAMP, loop-mediated amplification

RPA, recombinase polymerase amplification

SSBs, single-strand binding proteins

rtRPA, real-time RPA

LOD, limit of detection

IC_{RPA}, RPA assay internal control

B. atrophaeus, Bacillus atrophaeus

S. agalactiae, Streptococcus agalactiae

PPV, positive predictive value

NPV, negative predictive value

Ct, cycle threshold

POC, point-of-care

4.1 Introduction

Streptococcus agalactiae or group B streptococci (GBS) is one example of severe infectious diseases that cause sepsis and meningitis in neonates [1]. Between 20 and 40% of pregnant women may be colonized by GBS [2]. Vertical transmission from mother to the newborn accounts for 75% of neonatal GBS colonization and approximately 1% of these infants will develop early-onset GBS sepsis [3]. The 2002 Centers for Disease Control and Prevention (CDC) guidelines recommend prenatal screening of pregnant women at 35 to 37 weeks of gestation, with vaginal/anal culture and selective administration of intrapartum antibiotic prophylaxis to infected women for a minimum of 4 hours [4]. Implementation of these guidelines has reduced the incidence of early onset neonatal GBS disease from 1.5 to 0.3 per 1000 live births. The current gold standard method for GBS detection, culturing a vaginal/anal swab in selective medium broth [5], requires at least 48 hours for GBS identification (3). The culture method also has a lower clinical sensitivity compared to a molecular assay [6]. Furthermore, negative culture results for prenatal women may turn positive after labor [4]. Thus, in some cases the antibiotic treatment for GBS colonization may be either inappropriate or unnecessary [6]. A screening test that could detect women carrying GBS at labor could eliminate the need for prenatal screening at 35 to 37 week and reduce the risk of antibiotic prophylaxis for non-colonized women [3, 6]. The screening test must include a sample preparation method that ensures high recovery of nucleic acids and sufficient purity of clinical samples to control inhibitors [7, 8]. Comparative performance studies of automated extraction platforms have demonstrated the direct correlation between the performance of the extraction system and the imprecision of a molecular assay [7-9].

Real-time polymerase chain reaction (rtPCR) is useful for rapid and accurate GBS screening in pregnant women at time of delivery (10, 11). FDA-approved molecular tests based on rtPCR such as GBS GeneXpert, BD MAX GBS and BD GeneOhm™ StrepB assay can detect GBS and replace the standard culture [6, 10-15]. PCR is useful, one limitation is the need for a thermocycler that ensures rapid

heating/cooling temperature cycles [16]. Efforts to overcome PCR limitations have identified alternatives such as isothermal amplification techniques that do not require thermal cycling but instead rely on enzymatic activity for DNA/RNA synthesis [17]. Huy *et al.* [18] developed an isothermal amplification assay based on Loop-mediated amplification (LAMP) for the screening of GBS along with three other bacterial pathogens responsible for meningitis. Recently, the FDA cleared the Meridian Illumigene group B streptococcus DNA amplification assay, which uses LAMP to detect the pathogen in clinical samples and produces results in less than an hour [19].

Among the existing isothermal amplification techniques, recombinase polymerase amplification (RPA) operates between 25 °C and 42 °C. It features primer-recombinase complex in association with single-strand binding proteins (SSBs) to substitute heat cycles during the amplification process. RPA does not require an initial heat denaturation step to unwind dsDNA since the primer-recombinase complex along with SSBs ensure the unwinding and the stability of nucleic acid during the various exchange processes [20]. In addition, RPA primer design is simple and does not rely on sophisticated sequence design or on melting temperature considerations [21].

Owing to these advantages, we chose to evaluate an RPA assay as a potential point-of-care diagnostic method. Prior studies have applied RPA to aptamer ssDNA [22], positive control DNA [23], purified DNA [20, 24-28], human genomic DNA [20, 26, 29], double-stranded PCR product [30, 31], plasmid DNA [27, 28], cDNA [25, 26] and spiked plasma [25, 32], but none have tested RPA with human specimens. In contrast, we used actual human specimens in our study.

4.2 Materials and Methods

4.2.1 Target nucleic acids and clinical lysates

Fresh serial dilutions of purified genomic DNA of *Streptococcus agalactiae* strain ATCC 12973 of 15, 40, 80, 125, 250, 500 and 1000 genome copies were prepared as previously described [33] and used to determine the limit of detection (LOD) of RPA in a simplex assay. Fresh dilution of purified genomic DNA of *Bacillus atrophaeus* CCRI-9827 of 1000 genome copies was prepared to determine the LOD of RPA in a multiplex assay [34].

To determine the analytical specificity of rtRPA assay, purified genomic DNA from a variety of bacterial/fungal strains (table 4.1) naturally found in vaginal/anal samples were tested at 0.1 ng of DNA per reaction [33].

Vaginal/anal samples were collected from women in labor in accordance with a protocol approved by the ethical review board of Centre de recherche du CHU de Québec. The vaginal/anal samples were then eluted in Copan Transystem™ Liquid Stuart medium (Copan Italia International catalog no. 141C.USE), mechanically lysed and prepared according to the manufacturer's protocol for the BD GeneOhm™ Strep B assay kit. The lysates were screened for GBS for a first time by the rtPCR BD GeneOhm™ Strep B assay kit and the results recorded. These lysates were then stored at -80°C. Fifty of the frozen lysates were chosen for the comparative study between rtPCR and rtRPA.

An internal amplification control (IC_{RPA}) was included in the rtRPA assay with the clinical lysates. IC_{RPA} was a lysate of *Bacillus atrophaeus* spores. The latter were mechanically lysed with glass beads according to a homemade protocol that consisted of vigorous shaking of 50 µl spore dilution on a vortex mixer for 5 minutes followed by a brief centrifugation. The lysates were used at 1000 spores for each rtRPA amplification reaction [34]

4.2.2 Primers and Probes

For the rtPCR assay, the primers and probes for the target and internal control sequences were supplied in the BD GeneOhm™ Strep B assay kit [35].

For the rtRPA assay, the primers and probes (table 4.2) were designed according to the instruction manual using the TwistAmp™ exo kit (TwistDx). The forward and reverse primers as well as the probe for GBS were designed *in silico* to be specific for the *cfb* gene and generated a 234 bp product. The primers and probe for IC_{RPA} (table 4.2) were designed *in silico* to be specific for the *atpD* gene of *B. atrophaeus*. The generated product of the IC_{RPA} was 227 bp long.

4.2.3 Real-time PCR assay

The rtPCR procedure was performed according to the manufacturer's protocol for the BD GeneOhm™ Strep B assay kit. The amplification/detection of the samples was performed in a SmartCycler® instrument according to the SmartCycler®Dx Software Operator Manual.

4.2.4 rtRPA isothermal amplification assay

The real-time isothermal amplification procedure was performed using the reagents and protocols from the TwistAmp™ exo kit.

To evaluate the LOD and analytical specificity of the simplex assay, rtRPA was performed in a 25 µl total volume. First, a mastermix consisting of 29.5 µl rehydration buffer, 11.2 µl nuclease-free water, 2.1 µl forward primer (Sag59a) (420 nM), 2.1 µl reverse primer (cfbSag263) (420 nM) and 0.6 µl rtRPA probe (cfbSag159-E1-A1) (120 nM) were added to one freeze-dried reagent pellet and vortexed. The latter mix was separated into two reaction volumes of 22.75 µl each. One microliter of template DNA at the appropriate concentration was added to

each reaction: Template DNA for LOD analysis was GBS genomic DNA at a concentration corresponding to each of the serial dilutions. For analytical specificity analysis, template DNA was the genomic DNA of each of the chosen bacterial/fungal strains as well as GBS at 0.1 ng per amplification reaction. The reaction mixture was set on an ice-cold block to start a synchronized amplification in all the reaction tubes. The amplification reaction was initiated by the addition of 1.25 µl of magnesium acetate solution (14 mM) to each reaction. The reaction tubes were vortexed briefly and then incubated in a Rotor-Gene™ 6000 (Corbett Life Science,) at 39 °C for 30 min. Fluorescence measurements were taken every 30 seconds. For the LOD analysis, the number of replicates for each dilution was at least 12. The number of replicates for the analytical specificity analysis was 4.

To evaluate the LOD of the multiplex assay, rtRPA was performed in a 50 µl total volume. The reaction mixture was made of 29.5 µl rehydration buffer, 11.2 µl nuclease-free water, 1.05 µl of each forward primer (Sag59a and ABgl158b) (420 nM), 1.05 µl of each reverse primer (cfbSag263 and ABgl345c) (420 nM) and 0.3 µl of each rtRPA probe (cfbSag159-E1-A1 and ABgl220-E1-M2) (120 nM). One freeze-dried reagent pellet included in the kit was added to every reaction tube and vortexed. One microliter of GBS genomic DNA template at a concentration corresponding to each of the serial dilutions and 1 µl of *B. atrophaeus* genomic DNA of 1000 genome copies were added simultaneously to each reaction. The reaction mixture was set on an ice-cold block to start a synchronized amplification in all the reaction tubes. The amplification reaction was initiated by the addition of 2.5 µl of magnesium acetate solution (14 mM) to each reaction. The reaction tubes were vortexed briefly and then incubated in a Rotor-Gene™ 6000 at 39 °C for 30 min. Fluorescence measurements were taken every 30 seconds. The number of replicates for each dilution was 10.

For analysis of the frozen vaginal/anal lysates, rtRPA was performed in a 53 µl total volume. The reaction mixture was made of 29.5 µl rehydration buffer, 12.05 µl nuclease-free water, 1.05 µl of each forward primer (Sag59a and ABgl158b) (396 nM), 1.05 µl of each reverse primer (cfbSag263 and ABgl345c) (396 nM) and 0.3 µl

of each rtRPA probe (cfbSag159-E1-A1 and ABgl220-E1-M2) (113 nM). One freeze-dried reagent pellet included in the kit was added to every reaction tube and vortexed. Three microliters of lysate from every vaginal/anal sample and 1 µl of *B. atrophaeus* lysate of 1000 spores was added simultaneously to each reaction. The reaction mixture was set on an ice-cold block to start a synchronized amplification in all the reaction tubes. The amplification reaction was initiated by the addition of 2.65 µl of magnesium acetate solution (14 mM) to each reaction. The reaction tubes were vortexed briefly and then incubated in a Rotor-Gene™ 6000 at 39 °C for 40 min. Fluorescence measurements were taken every 30 seconds.

4.2.5 Statistical analysis

LOD data for the simplex and multiplex assays were statistically analyzed with homebrew software Lotlod (version 1.2.2) that used R software (version 2.14.1) [36] to determine LOD with their 95% confidence interval from a logistic regression model.

4.3. Results

4.3.1 Limit of detection

For both the simplex and multiplex assays, rtRPA was able to detect as little as 15 genomic copies in 50% of cases (Supplemental data table 4.4.). For the simplex assay, we determined with Lotlod that the LOD of the rtRPA for GBS was 98±20 genomic copies (95% CI). In the presence of IC_{RPA} genomic DNA, the LOD for the multiplex assay was 100±20 genomic copies (95% CI). The time threshold varied among replicates of a same dilution as well as between other dilutions (Supplemental data figure 4.2).

4.3.2 Analytical specificity

The rtRPA assay did not detect the genomic DNA of the 13 following bacteria: *Lactococcus lactis*, *Streptococcus pyogenes*, *Bifidobacterium breve*, *Anaerococcus lactolyticus*, *Acinetobacter baumannii*, *Bordetella pertussis*, *Bacteroides fragilis*, *Mobiluncus mulieris, Mobiluncus curtisii* subsp. *holmesii, Trichomonas vaginalis, Gardnerella vaginalis, Chlamydia trachomatis* and *Neisseria gonorrhoeae* (table 4.1). It also did not detect the genomic DNA of 2 fungi, which were *Candida albicans* and *Candida krusei* (table 4.1). These bacterial and fungal strains are naturally found in the vaginal/anal flora. These results show the analytical specificity of the rtRPA assay with our designed set of primers and probe (Sag59a, cfbSag263 and cfbSag159-E1-A1, respectively) to GBS (table 4.2).

4.3.3 GBS screening of frozen clinical lysates with rtPCR and rtRPA

Among the 50 frozen vaginal/anal lysates, 25 were identified as positive and 25 identified as negative for GBS by the real-time PCR with the BD GeneOhm^{TM} Strep B assay kit. With the latter assay the time to result was approximately 45 minutes. The analysis made by the SmartCycler®Dx Software showed no discordant nor unresolved samples. A sample was considered as unresolved if the IC_{RPA} failed to amplify.

Compared to the results obtained by rtPCR, 5 clinical samples were unresolved by rtRPA. Upon re-testing, 1 became a false negative and 4 became true negatives for GBS since the IC_{RPA} was detected in all 5 samples. Among the 25 PCR positive samples, 24 were positive with rtRPA, showing a clinical sensitivity of 96 percent (table 4.3). Among the 24 GBS positive samples, the time threshold was less than 10 min for 15 samples, less than 15 min for 8 samples and 15.5 min for one sample. The mean time threshold for GBS positive samples was 9.0 min with a standard deviation of 2.9 min. For IC_{RPA} the mean time threshold for GBS positive samples was of 11.5 min with a standard deviation of 2.6 min (Supplemental data figure 4.3A).

With respect to the rtPCR negative samples, rtRPA agreed 100% with the rtPCR results (table 4.3). Among the 25 GBS negative samples, the time threshold for IC_{RPA} was less than 10 min for 3 samples, less than 15 min for 15 samples, and greater than 23 min for 7 samples (Supplemental data figure 4.3B). For IC_{RPA} the mean time threshold for GBS negative samples was 16.0 min with a standard deviation of 8.3 min. The positive predictive values (PPV) and negative predictive values (NPV), for the rtRPA assay (table 4.3) showing a PPV of 100% and a NPV of 96%.

4.3.4 Speed of reaction of rtRPA versus rtPCR

In this study, we also evaluated the difference in amplification time-to-result between rtRPA and rtPCR by calculating the mean time threshold, which was the time (min) at which an amplification signal was detected. For this purpose, we converted the cycle threshold obtained by SmartCycler for a whole run into minutes so that it matched the time threshold for an rtRPA assay (data not shown). We then calculated the mean with the corresponding standard deviation of the time threshold for each of the 24 GBS positive clinical lysates obtained with rtRPA and rtPCR. We observed that the mean time threshold for the same positive clinical samples for the rtRPA assay (9 min) betters the rtPCR assay (29 min) in time-to-result by approximately 20 min (figure 4.1). In addition, since both rtPCR and rtRPA used the same sample preparation method, the total time-to-answer was determined by the amplification/detection time. For rtRPA assay, it was at least 20 min faster than rtPCR with all the steps carried out at a single temperature.

4.4 Discussion

In this study, we evaluated the clinical performance of isothermal amplification technique rtRPA compared to a reference rtPCR assay. We chose the RPA method for two reasons. First, RPA allows a real-time detection of amplification via fluorescent probe. Second, RPA freeze-dried reagents pellets are stable at room temperature for days, which makes its application easier in point-of-care [26, 27].

In our study, the LOD for the rtRPA simplex assay was 98±20 and 100±20 genomic copies (95% CI) for the multiplex assay. The presence of 1000 genomic copies of the IC_{RPA} in the same reaction tube with the template did not impact the limit of detection of the GBS template. The reported analytical sensitivity of the BD GeneOhm™ strep B rtPCR assay is of 10 to 50 genome copies. We expect that the analytical sensitivity of the rtRPA assay could be improved by providing a proper mixing of RPA reagents after a few minutes of amplification. This has worked with the TwistAmp™ exo kit protocol [37], especially when working with low DNA concentration. Lutz *et al.*[30] ensured a proper mixing of the amplification reagents with a modified Rotor-Gene 2000 by removing the reaction tubes from the instrument after 3 min of amplification for an additional vortexing step. The reaction tubes were then placed again in the instrument for real-time detection. However, we were not able to add a mixing step after 3 min with the Rotor-Gene 6000, and real-time monitoring could not be interrupted without disruption of fluorescence acquisition. This may explain both the LOD and the variability in time threshold between replicates of different dilution samples that we observed.

We designed an rtRPA assay to specifically amplify a sequence of the *cfb* gene for GBS. Amplification did not take place with the tested bacteria and fungi naturally found in vaginal/anal flora. The high analytical specificity of the rtRPA makes the assay amenable to clinical applications.

In clinical applications, molecular methods are generally subject to the problem of contaminants and inhibitors from crude samples [38]. Some of our preliminary results obtained with 15 vaginal/anal crude samples spiked with 1000 copies of

GBS genomic DNA showed that an initial heat denaturation step at 95 °C for 2 min after sample lysis may reduce the inhibition and enhance speed of reaction for rtRPA (data not shown). As with rtPCR, the omission of heating at 95°C to denature PCR inhibitors decreased the efficiency of detection of *B. atrophaeus* DNA [34]. Our study with the 50 frozen clinical lysates demonstrated a good performance of rtRPA compared to the rtPCR. The only false negative sample obtained was not inhibitory for *B. atrophaeus* IC_{RPA}, suggesting that the RPA reaction was not inhibitory for GBS either. This led us to assume that the bacterial load of this clinical lysate was probably below the LOD of rtRPA. When we compared the cycle thresholds (Ct) obtained with SmartCycler for the 50 clinical lysates tested, we found that the false negative sample was among the samples with high Ct, demonstrating that it was among the lowest bacterial load samples (data not shown). Because mixing after few minutes of amplification is recommended, the false negative sample may be explained by the performance of RPA in the absence of proper mixing. Among the GBS negative clinical lysates, 7 were shown to be inhibitory since the IC_{RPA} amplification signal was delayed by 10 to 20 min compared to other samples. In addition, we showed the feasibility of multiplexing with rtRPA assay in which two different DNA targets, GBS and *B. atrophaeus*, were simultaneously amplified with multiplex sets of primers and probes. Different amplicons were generated and detected in the same reaction tube at the same time. Therefore, *B. atrophaeus* could serve as internal control as well as process control [34].

Considering GBS from a clinical perspective, the PPV and NPV values show that a positive test with rtRPA is a strong indication that GBS colonization is present (100%). However, with a negative test it is possible that GBS colonization could be undetected (4%). It will be important to verify the performance of rtRPA-GBS assay with fresh vaginal/anal swabs collected directly from women in labor.

The rtRPA assay shows some advantages over rtPCR. It is faster in terms of runtime and obviates the need for a sophisticated instrument for thermal management. Combined with the avoidance of labor costs due to the requirements

for trained personnel in a certified laboratory, a POC RPA assay becomes more cost effective. The BD GeneOhm™ strep B rtPCR assay used in this study costs approximately $35 per test just for disposable and $48 600 for the SmartCycler® instrument. We predict that an RPA-based POC assay would cost approximately $10 per disposable and $10 000 or less for a dedicated instrument. We consider this work promising for a future application of an RPA method as an alternative tool in clinical settings for the screening and detection of multiple infectious agents.

Acknowledgments

We thank, Marthe Bernier, Martin Gagnon and Dominique K. Boudreau for providing us with useful technical advices to accomplish this work. We further thank Eric Martel for providing us with the Lotlod software for the statistical analysis.

References

[1] Oh W. Early Onset Neonatal Group B Streptococcal Sepsis. Am J Perinatol. 2013.

[2] Boyer KM, Gotoff SP. Prevention of early-onset neonatal group B streptococcal disease with selective intrapartum chemoprophylaxis. N Engl J Med. 1986;314:1665-9.

[3] de-Paris F, Machado ABMP, Gheno TC, Ascoli BM, de Oliveira KRP, Barth AL. Group B *Streptococcus* detection: comparison of PCR assay and culture as a screening method for pregnant women. Braz J Infect Dis. 2011;15:323-7.

[4] Schrag S, Gorwitz R, Fultz-Butts K, Schuchat A. Prevention of perinatal group B streptococcal disease. Revised guidelines from CDC. MMWR Recomm Rep. 2002;51:1-22.

[5] Verani JR, McGee L, Schrag SJ. Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010. MMWR Recomm Rep. 2010;59:1-36.

[6] Davies HD, Miller MA, Faro S, Gregson D, Kehl SC, Jordan JA. Multicenter study of a rapid molecular-based assay for the diagnosis of group B Streptococcus colonization in pregnant women. Clin Infect Dis. 2004;39:1129-35.

[7] Verheyen J, Kaiser R, Bozic M, Timmen-Wego M, Maier BK, Kessler HH. Extraction of viral nucleic acids: comparison of five automated nucleic acid extraction platforms. J Clin Virol. 2012;54:255-9.

[8] Yang G, Erdman DE, Kodani M, Kools J, Bowen MD, Fields BS. Comparison of commercial systems for extraction of nucleic acids from DNA/RNA respiratory pathogens. J Virol Methods. 2011;171:195-9.

[9] Schuurman T, de Boer R, Patty R, Kooistra-Smid M, van Zwet A. Comparative evaluation of in-house manual, and commercial semi-automated and automated DNA extraction platforms in the sample preparation of human stool specimens for a *Salmonella enterica* 5'-nuclease assay. J Microbiol Methods. 2007;71:238-45.

[10] Park JS, Cho DH, Yang JH, Kim MY, Shin SM, Kim EC, et al. Usefulness of a Rapid Real-time PCR Assay in Prenatal Screening for Group B Streptococcus Colonization. Ann Lab Med. 2013;33:39-44.

[11] Gavino M, Wang E. A comparison of a new rapid real-time polymerase chain reaction system to traditional culture in determining group B streptococcus colonization. Am J Obstet Gynecol. 2007;197:388 e1-4.

[12] Edwards RK, Novak-Weekley SM, Koty PP, Davis T, Leeds LJ, Jordan JA. Rapid group B streptococci screening using a real-time polymerase chain reaction assay. Obstet Gynecol. 2008;111:1335-41.

[13] Schwartz J, Robinson-Dunn B, Makin J, Boyanton BL, Jr. Evaluation of the BD MAX GBS assay to detect Streptococcus group B in LIM broth-enriched antepartum vaginal-rectal specimens. Diagn Microbiol Infect Dis. 2012;73:97-8.

[14] Gray JW, Milner PJ, Edwards EH, Daniels JP, Khan KS. Feasibility of using microbiology diagnostic tests of moderate or high complexity at the point-of-care in a delivery suite. J Obstet Gynaecol. 2012;32:458-60.

[15] Daniels J, Gray J, Pattison H, Roberts T, Edwards E, Milner P, et al. Rapid testing for group B streptococcus during labour: a test accuracy study with evaluation of acceptability and cost-effectiveness. Health Technol Assess. 2009;13:1-154, iii-iv.

[16] Craw P, Balachandran W. Isothermal nucleic acid amplification technologies for point-of-care diagnostics: a critical review. Lab Chip. 2012;12:2469-86.

[17] Gill P, Ghaemi A. Nucleic acid isothermal amplification technologies - A review. Nucleosides Nucleotides Nucleic Acids. 2008;27:224-43.

[18] Huy NT, Hang le TT, Boamah D, Lan NT, Van Thanh P, Watanabe K, et al. Development of a single-tube loop-mediated isothermal amplification assay for detection of four pathogens of bacterial meningitis. FEMS Microbiol Lett. 2012;337:25-30.

[19] Meridian Bioscience. Products: illumigene® Molecular Diagnostic System

[20] Piepenburg O, Williams CH, Stemple DL, Armes NA. DNA detection using recombination proteins. PLoS Biol. 2006;4:1115-21.

[21] TwistDx. Instruction Manuals.

[22] Loo JFC, Lau PM, Kong SK. An aptamer-based bio-barcode assay with isothermal recombinase polymerase amplification for cytochrome-*c* detection and anti-cancer drug screening. Talanta. 2013;115:159-65.

[23] Hakenberg S, Hugle M, Weidmann M, Hufert F, Dame G, Urban GA. A phaseguided passive batch microfluidic mixing chamber for isothermal amplification. Lab Chip. 2012;12:4576-80.

[24] Shen F, Davydova EK, Du WB, Kreutz JE, Piepenburg O, Ismagilov RF. Digital Isothermal Quantification of Nucleic Acids via Simultaneous Chemical Initiation of Recombinase polymerase amplification Reactions on SlipChip. Anal Chem. 2011;83:3533-40.

[25] Euler M, Wang Y, Heidenreich D, Patel P, Strohmeier O, Hakenberg S, et al. Development of a panel of Recombinase polymerase amplification assays for the detection of biothreat agents. J Clin Microbiol. 2013;51:1110-7.

[26] Euler M, Wang YJ, Nentwich O, Piepenburg O, Hufert FT, Weidmann M. Recombinase polymerase amplification assay for rapid detection of Rift Valley fever virus. J Clin Virol. 2012;54:308-12.

[27] Euler M, Wang YJ, Otto P, Tomaso H, Escudero R, Anda P, et al. Recombinase polymerase amplification Assay for Rapid Detection of Francisella tularensis. J Clin Microbiol. 2012;50:2234-8.

[28] Boyle DS, Lehman DA, Lillis L, Peterson D, Singhal M, Armes N, et al. Rapid detection of HIV-1 proviral DNA for early infant diagnosis using recombinase polymerase amplification. MBio. 2013;4.

[29] Shin Y, Perera AP, Kim KW, Park MK. Real-time, label-free isothermal solid-phase amplification/detection (ISAD) device for rapid detection of genetic alteration in cancers. Lab Chip. 2013;13:2106-14.

[30] Lutz S, Weber P, Focke M, Faltin B, Hoffmann J, Muller C, et al. Microfluidic lab-on-a-foil for nucleic acid analysis based on isothermal recombinase polymerase amplification (RPA). Lab Chip. 2010;10:887-93.

[31] Rohrman BA, Richards-Kortum RR. A paper and plastic device for performing recombinase polymerase amplification of HIV DNA. Lab Chip. 2012;12:3082-8.

[32] Escadafal C, Paweska JT, Grobbelaar A, le Roux C, Bouloy M, Patel P, et al. International external quality assessment of molecular detection of Rift Valley fever virus. PLoS Negl Trop Dis. 2013;7:e2244.

[33] Ke D, Menard C, Picard FJ, Boissinot M, Ouellette M, Roy PH, et al. Development of conventional and real-time PCR assays for the rapid detection of group B streptococci. Clin Chem. 2000;46:324-31.

[34] Picard FJ, Gagnon M, Bernier MR, Parham NJ, Bastien M, Boissinot M, et al. Internal control for nucleic acid testing based on the use of purified *Bacillus atrophaeus* subsp. *globigii* spores. J Clin Microbiol. 2009;47:751-7.

[35] Jordan JA, Hall G, Davis T. Multicenter Study Evaluating Performance of the Smart Group B Streptococcus (GBS) Assay Using an Enrichment Protocol for Detecting GBS Colonization in Patients in the Antepartum Period. J Clin Microbiol. 2010;48:3193-7.

[36] R Project. The R Project for Statistical Computing.

[37] TwistDx. Products: TwistAmp exo, real-time fluorescent DNA detection.

[38] Sakallah SA. Molecular diagnostics of infectious diseases: state of the technology. Biotechnol Annu Rev. 2000;6:141-61.

Table 4.1. Bacterial/fungal strains tested for specificity

Strain	ATCC *n*	rtRPA result
Streptococcus agalactiae	12973	+
Lactococcus lactis	19435	-
Streptococcus pyogenes	19615	-
Bifidobacterium breve	15700	-
Anaerococcus lactolyticus	51172	-
Acinetobacter baumannii	19606	-
Bordetella pertussis	9797	-
Bacteroides fragilis	25285	-
Mobiluncus mulieris	35243	-
Mobiluncus curtisii subsp. *holmesii*	35242	-
Trichomonas vaginalis	30001	-
Gardnerella vaginalis	14019	-
Chlamydia trachomatis	VR-902B	-
Neisseria gonorrhoeae	43069	-
Candida albicans	10231	-
Candida krusei	34135	-

Table 4.2. Primers and probes used in rtRPA assay

Name[a]	Sequence (5'-3')	Source
Sag59a	TTTCACCAGCTGTATTAGAAGTACATGCTGATC	Integrated DNA Technologies
cfbSag263	ACTGTCTCAGGGTTGGCACGCAATGAAGTC	Integrated DNA Technologies
cfbSag159-E1-A1	GCTTGATCAAGATAGCATTCAGTTGAGAAA (FAMdT)(THF)(BHQ1dT)CAAAGATA ATGTTCAGGG(Spacer-C3)[b]	Biosearch Technologies
ABgl158b	AGAGGTCGCACTTCATTTAGGCGACGATACT	Integrated DNA Technologies
ABgl345c	ACGGAGCTTGTCTGTGAATCGGATCTTTCTC	Integrated DNA Technologies
ABgl220-E1-M2	GCGCGGAATGGAAGCGGTGGACCAAGGTTC (M2dT)C(THF)(BHQ2dT)ATTTCAGT GCCGGTT(phosphate)[c]	Biosearch technologies

[a] Sag59a/cfbSag263, GBS forward/reverse primers; cfbSag159-E1-A1, GBS rtRPA probe; ABgl158b/ABgl345c, IC_{RPA} forward/ reverse primers; ABgl220-E1-M2, IC_{RPA} rtRPA probe.
[b] FAMdT, fluorescein linked to thymidine; THF, tetrahydrofuran spacer; BHQ1dT, Black Hole Quencher 1 linked to thymidine; 3' Spacer-C3 blocking elongation.
[c] M2dT, Texas Red linked to thymidine; THF, tetrahydrofuran spacer; BHQ2dT, Black Hole Quencher 2 linked to thymidine; 3' phosphate blocking elongation.

Table 4.3. Clinical performance of rtRPA

		BD GeneOhm™ StrepB (reference assay)		
		Positive	Negative	%
rtRPA	Positive	24 (TP)[a]	0 (FP)[b]	100 (PPV)[e]
	Negative	1 (FN)[c]	25 (TN)[d]	96 (NPV)[f]
	%	96 (Sensitivity)	100 (Specificity)	

[a] True Positive (TP); [b] False Positive (FP); [c] False Negative (FN); [d] True Negative (TN); [e] Positive predictive value (PPV); [f] Negative predictive value (NPV).

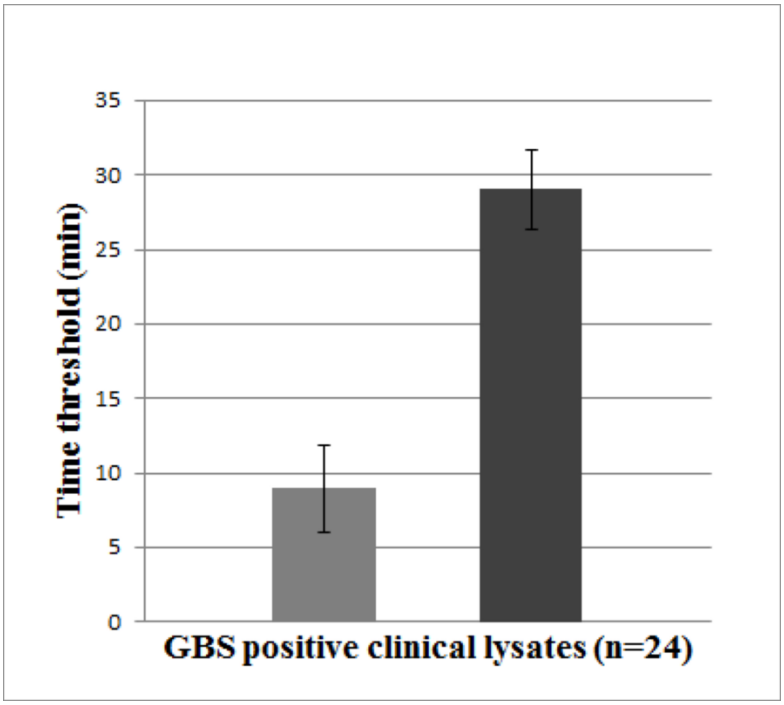

Figure 4.1. Difference in amplification time-to-result between rtPCR and rtRPA for 24 samples

Gray bar represents the mean time threshold of the GBS positive sample amplified by rtRPA with the TwistAmp™ exo kit. Black bar represents the mean time threshold of the same GBS positive sample yet amplified by rtPCR with BD GeneOhm™ StrepB assay. Standard deviation is also plotted for each of the mean time bars.

Supplemental data tables

Table 4.4. rtRPA LOD data with and without IC_{RPA}

GBS DNA Dilutions	*n* of positives, simplex assay (total = 12)	*n* of positives, multiplex assay (total = 10)
15	6	5
40	9	6
80	10	8
125	12	10
250	12	10
500	12	10
1000	12	N/A

Supplemental data figures

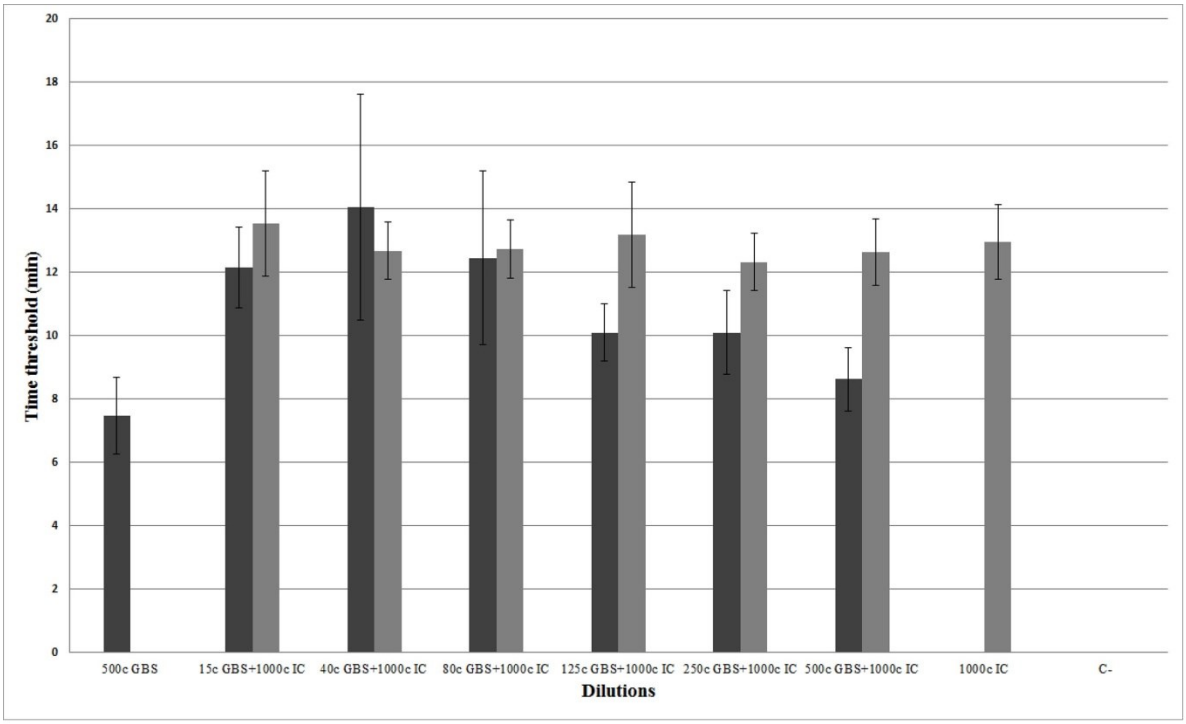

Figure 4.2. Variability of time threshold in rtRPA

Black bar represents the mean time threshold of each GBS genomic DNA dilution amplified by rtRPA with the TwistAmp^{TM} exo kit. Gray bar represents to the mean time threshold of 1000 copies of IC_{RPA} genomic DNA. Standard error of the mean shows the variability of time threshold for each set of dilution samples. IC, internal control (*i.e.* IC_{RPA}); C-, negative no template control.

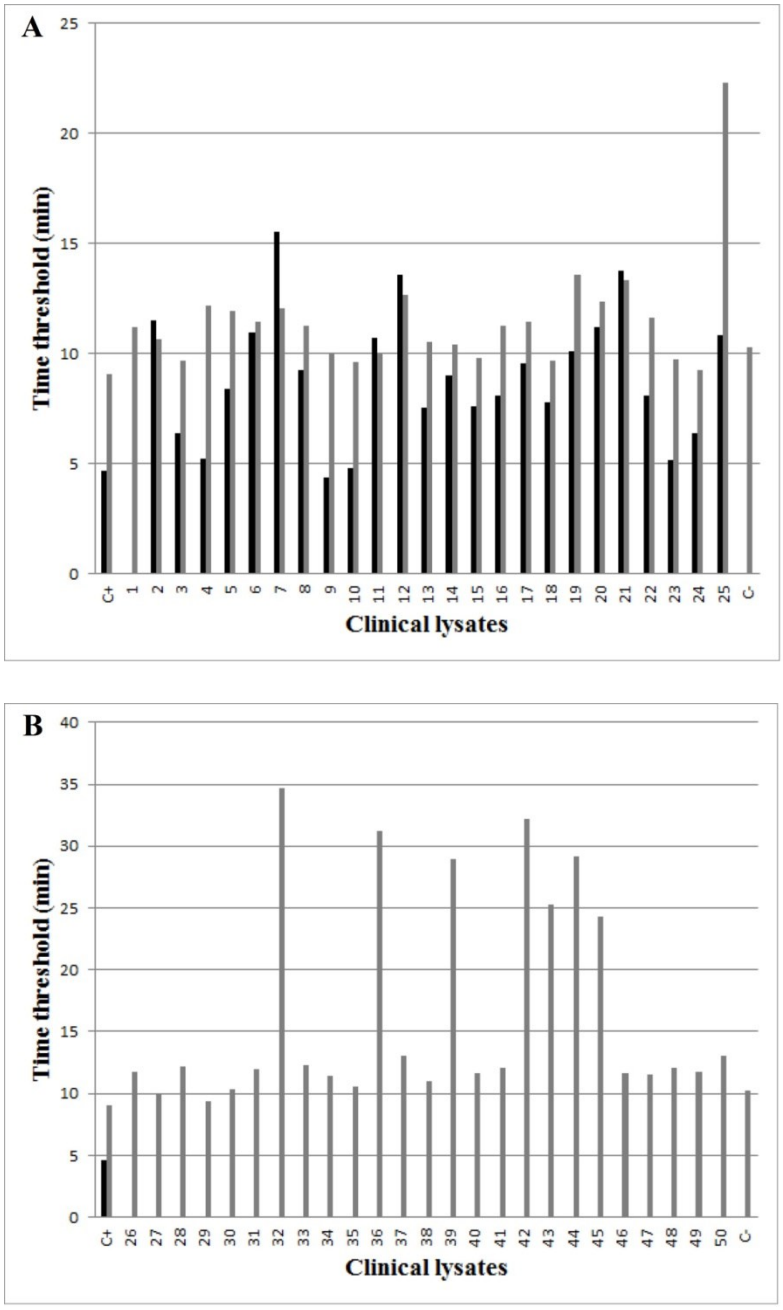

Figure 4.3. Performance of rtRPA assay with 50 frozen vaginal/anal lysates

Time threshold for amplification signal obtained by Rotor-Gene normalized fluorescence curves for (A) the first set of GBS positive samples and (B) second set of GBS negative samples as determined with rtRPA with the TwistAmp^{TM} exo kit. Black bar represents signal of amplification for GBS positive sample while gray bar is the amplification signal for IC_{RPA}. C+, GBS genomic DNA at 1000 copies; C-, lysate of *B. atrophaeus* at 1000 spores.

Chapter

V. Preliminary results and challenges of a microfluidic miniaturized sample-to-answer cartridge

5.1 Introduction

Point-of-care tests (POCTs) promise to bring diagnostic assays close to the patient (Hartman, Ruiz *et al.* 2013) with minimal intervention by laboratory personnel (Moore 2013) thus allowing rapid time to result. This concept has expanded to include any test that can be performed rapidly outside hospitals and centralized laboratories (Moore 2013). POCTs are valuable for resource-limited settings as well as for developed countries since they can offer accessible testing for infectious diseases in a portable home-based format (Hartman, Ruiz *et al.* 2013). The World Health Organization has developed attributes required for POCTs defined as "ASSURED" criteria for affordable, sensitive, specific, user-friendly, rapid/robust, equipment-free or minimal, and delivered to the greatest need (Mabey, Peeling *et al.* 2004; Urdea, Penny *et al.* 2006). This calls for a radical simplification of POCTs equipment (Bearinger and Dugan 2011) which in turn is translated into reduced power consumption, smaller sample volume requirements, and faster heat transfer properties. The challenge is that POCTs are expected to perform as accurately as centralized laboratory-based tests (Mabey, Peeling *et al.* 2004). Efforts have been made to develop miniaturized and disposable POCTs ready for diagnostics near patient. However, most POCTs used for infectious diseases are based on lateral flow immunochromatography recognition methods for which performance is dependent on disease prevalence, personnel qualifications, and quality control assessment (Sturenburg and Junker 2009).

Simplification of POCTs requires less hands-on manipulation which means that all biological steps from sample preparation to target amplification/detection must be automated. In this chapter, a microfluidic cartridge, named blade, is paired with an automated NADTech instrument for real-time detection of fluorescence signals. This automated centripetal platform was configured to be simple and portable for the rapid and specific detection of infections near the patient. The blade was inspired from previous work that employed a centrifugal compact disc (CD) device for rapid and automated processing of nucleic acid samples (Siegrist, Gorkin *et al.* 2011; Roy, Stewart *et al.* 2014). As in the case of the CD, the blade was designed

to fulfill 3 distinct operational steps: sample preparation, target amplification and signal detection. These steps are typically required for POCTs (Hartman, Ruiz *et al.* 2013). Except for the initial sample loading, these labor-intensive manual steps are eliminated. Consequently, the time required for a total analysis on the blade (~30 min) is reduced compared to the time required for performing the same steps manually (> 60 min). In addition, the system is automated thus reducing potential human errors. The blade configuration works with lyophilized reagents and small liquid volumes (µl scale) which helps keep cost down and facilitates higher throughput.

The blade performs isothermal amplification of nucleic acids by recombinase polymerase amplification technology (RPA) (Piepenburg, Williams *et al.* 2006). The objective of this work was to automate the previously described RPA-GBS assay (chapter IV) in the blade while using the dedicated NADTech instrument. The blade and NADTech instrument were tested and verified using both *Streptococcus agalactiae* genomic DNA (Group B streptococci, GBS) and *Bacillus atrophaeus* subsp. *globigii* spores.

This work was achieved by a multidisciplinary collaboration. The design of the blade was generated by experts in materials and microfluidics of the National Research Council Canada (NRC). The design of NADTech instrument was executed by David Béliveau-Viel an expert in engineering at the *Centre d'optique, photonique et laser de l'Université Laval (COPL)*. My contribution was in the first place in the configuration of the blade. This includes defining specific volumes of RPA reagents and their compartmentalisation in separate operational chambers. In the second place, it was in determining the entire biological process conditions that included the duration and temperature for sample preparation, amplification, and fluorescence acquisition. All experiments were conducted with the assistance of David Béliveau-Viel. In the third place, it was in writing the first draft of the manuscript.

Herein, we present preliminary results for a proof-of-concept of an automated sample-to-answer platform for nucleic acid isothermal amplification using RPA technology and real-time detection of fluorescence signals.

5.2 Materials and Methods

5.2.1 RPA reagents

For the blade assays, both TwistAmp® basic and exo kits were used. A forty microliter of rehydration solution was formed in nuclease-free water consisting of 1x buffer (TwistAmp®), 420 nM forward and reverse primers, 120 nM exo probes (only when using TwistAmp® exo kit for real time detection), and 14 mM magnesium acetate. The primers and probes used were the same as in chapter IV, table 4.2. This rehydration solution is then manually loaded into the dedicated chamber (C) (figure 5.1) before each assay. RPA lyophilized pellet was used in its dried format and confined directly inside the blade (chamber (D), figure 5.1) for subsequent manipulations.

5.2.2 Lysis solution

For sample preparation, a home-made lysis solution (Picard and Menard 2009) was injected in the dedicated chamber of the blade (chamber (A), figure 5.1). It consisted of a 60 µl of 0.077g two size silica beads (150 to 212 µm, Sigma G-1145/1152) in Milli-Q water with the addition of 3 g of polyvinylpyrrolidone, a component of nucleic acid extraction buffers (Sigma P0930).

5.2.3 Conception of the blade

The design was initially made to perform 3 key steps that are sample preparation, nucleic acid amplification, and detection. The blade went through several design iterations. Each new design was subjected to some adjustment and prototyping. The final generation consisted of a thin Zeonor® plastic slide covered with a thermoplastic elastomer layer embossed with microfluidic channels and chambers. To this end, using the Cyclo-olefin polymer Zeonor® had many advantages. First, it had high transparency and flowability for molding or hot embossing. It also had high thermostability and low auto-fluorescence that is an important criterion in avoiding background noise especially when using fluorescent probes for real-time detection. Furthermore, bonding of the elastomeric layer to the Zeonor® plastic slide was achieved by contact and heating at 80°C for 1 hour.

The blade was designed to have 5 biological chambers with different predefined volume capacity, interconnected via microfluidic channels, and adapted to RPA technology. Figure 5.1 depicts the detailed structure of the blade and the function of each chamber. In brief, the first chamber (A) was assigned for sample preparation that consisted of a homogenization and mechanical lysis steps. Chamber (A) receives 100 µl of sample manually loaded. The second chamber (B) is connected to (A) via the capillary burst valve 1 retaining 10 µl of lysate. Another capillary valve 2 connects (B) to chamber (C) that is preloaded manually with 40 µl of RPA rehydration solution. Chamber (C) is connected to the amplification/detection chamber (D) via a siphon valve. The chamber (D) is prefilled originally with RPA lyophilized reagent pellet. The fifth chamber (E) is the waste chamber collecting excess fluids. The operation of the blade will be detailed in section 5.2.5.

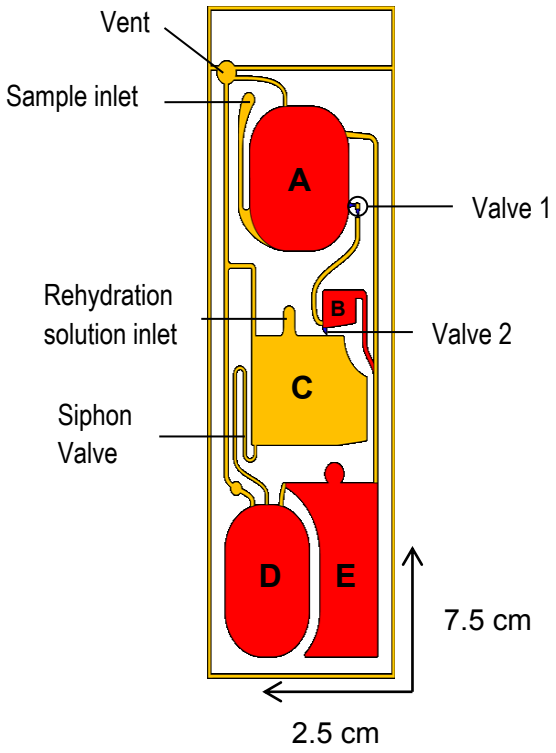

Figure 5.1. Schematic representation of the structure of the blade

Five microfluidic chambers (A to E) connected via channels. (A), sample-preparation chamber; (B), aliquot chamber; (C), rehydration solution chamber; (D), amplification/detection chamber; (E), waste chamber.

5.2.4 Development of NADTech instrument

Three generations of the instrument were produced to improve performance, autonomy and reduce size. The final generation of the instrument was entirely autonomous, capable of launching predefined sequences of centrifugation, heating and detection of fluorescent signals (table 5.1). It was able to show the results on a screen and to export fluorescence curves to the computer.

The apparatus measuring 20 x 20 cm accommodated 2 blades per run. The incubation inside the instrument was regulated by heated air influx via a NiChrome wire reaching up to 120°C. Magnets were integrated in the enclosure floor encored beneath each blade so that they drove the movement of ferromagnetic disc inside

the blade with each centrifugation sequence (Chamber (A), figure 5.1). The instrument was conceived for reading 2 fluorescent dyes: fluorescein (494-515 nm) and Texas Red® (583-603 nm). The excitation and detection of each dye were enabled with a light emitting diode as a source and a photodiode as the detector. During RPA incubation, the amplification/detection chamber of the blade was held above the light source less than 1 second for fluorescence reading. The reading took place every 30 seconds. Fluorescence levels were displayed on a liquid crystal screen. The positivity of the sample was determined by analyzing the increase in the fluorescence signal. Fluorescence values were registered and downloaded easily to a computer using a USB or Bluetooth connection to produce a graph.

Figure 5.2. NADTech instrument

The automated apparatus can hold two blades at a time. The amplification/detection process started with a push of a button and the result for each blade was visualized on the screen. Fluorescence reading was made in two channels herein, fluorescein (FAM) and Texas Red®.

5.2.5 Blade operation with NADtech instrument

The operation of the blade began by manually loading chambers (A) and (B) with 100 µl of sample and 40 µl of RPA rehydration solution respectively. Chambers inlets were then taped to avoid evaporation during instrument operation sequence. Two blades were tested per run and the operation sequences started when pressing the start button of the instrument. Instrument operation sequences are detailed in table 5.1 according to the type of assay executed. However, the blade and the instrument were designed to fulfill the following functions. First, homogenization of the sample in chamber (A) was done to allow equal distribution of target cells with silica beads. Lysis of target cells was then initiated by speeding up the centrifugation which in turn accelerated the movement of the ferromagnetic disc within the chamber walls. This caused beads beating to shear cells thus liberating target nucleic acids. The lysis step was executed at an elevated temperature of 120°C (It was determined that an influx of air at 120°C for 5-7 min would bring fluid temperature close to 95°C) to deactivate contaminants. A rapid cool down brought fluid temperature to 20°C which is crucial to avoid loss of activity of RPA lyophilized pellet at elevated temperatures, >42°C (TwistDx 2015a). Accelerating centrifugation (from 600 to 1100 RPM) allowed metering so that a precise volume of lysate (*i.e.* 10 µl) flew through valve 1 filling chamber (B). Another round of centrifugation acceleration (from 1100 to 2200 RPM) filled chamber (C) with the lysate. To fill chamber (D), a deceleration in centrifugation (from 2200 to 500 RPM) primed the siphon valve. Consequently, chamber (D) received 50 µl of lysate in rehydration solution that reconstituted RPA lyophilized pellet. The incubation was performed at 39°C for 30 min with fluorescence reading every 30 sec. Fluorescence acquisition was done in both FAM (GBS detection) and Texas Red® (*Bacillus* detection) channels.

Table 5.1. Instrument operation sequences for different blade functions

		Conditions	
Operation sequences/Functions	Duration (sec)	Centrifugation speed (RPM)	Temperature (°C)
Homogenization/Lysis	420	600	120
Cool down	300		20
Metering	40	1100	
Filling chamber (C)	30	2200	
Filling chamber (D)	30	500	
Incubation/ Fluorescence reading	1800	400	39
Turnaround time (sec)*	**2620 (~44 min)**		

* Turnaround time depends on assay conditions. When no lysis step is required, sample homogenization is shorter taking 300 sec without heating up the cartridge, thus eliminating the cool down step. Moreover, when using RPA basic reagents that does not contain fluorescent probe, fluorescence reading is also omitted. In this case, the turnaround time took 29 min.

5.2.6 Validation assays of the blade and NADTech instrument

More than 150 prototype blades were generated from NRC and served for different assays during this project. Before conducting experiments with biological samples, we validated that the blades were microfluidically functional, capable of performing the above mentioned steps, and did not have any fluid draining problems. The following sections focus on few representative preliminary results obtained during this doctoral project.

5.2.6.1 Amplification verification assays

To assess the amplification yield in the blade, 10 prototypes preloaded with RPA basic lyophilized pellet were used to quantitatively measure the amplicon yield on agarose gel electrophoresis. On the other hand, 9 other blades were preloaded with the real-time RPA-exo reagent pellet to assess fluorescence signals acquisition with the instrument. All 19 blades were used for the amplification of GBS genomic DNA.

Eight RPA-basic blades were loaded with 100 µl of sample solution contained 10^6 copies of GBS genomic DNA. Two negative control blades were loaded with 100 µl of 1x TE buffer (10 mM Tris, pH 8.0 with HCl and 1 mM EDTA). As described previously in section 5.2.5, the blades were taped and subjected to the operation sequences without including a lysis and a fluorescence reading step (table 5.1). Monitoring of the amplicons was made from RPA reaction mixture collected by a fine needle syringe to pierce chamber (D) elastomeric surface and transfer the volume into a 0.2 mL Eppendorf™ tube. RPA amplicons were purified with QIAquick PCR Purification Kit (QIAGEN Inc., Toronto, ON, Canada) according to the manufacturer's protocol. Purified amplicons were visualized after 40 min migration on a 2% agarose gel electrophoresis with 0.25 µg/mL of ethidium bromide in Tris-borate-EDTA buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3). Amplicon sizes were verified with a 1-kb molecular weight DNA ladder (Invitrogen, Carlsbad, CA, USA). The intensity of specific amplicon band was quantified according to the instruction manual of AlphaImager™ 2000 (Alpha Innotech Corporation, San Leandro, CA, USA).

For the RPA-exo blades, the experimental protocol was similar except the addition of RPA-exo probe in rehydration solution (section 5.2.1). The only change in the operation sequence was in not including a lysis step. Fluorescence was acquired in FAM channel.

5.2.6.2 Sample-to-answer verification assays

To attempt the efficiency of the lysis step in the blade, *B. atrophaeus* spores (CCRI-9827) were used. In addition, these spores served as internal and process control as demonstrated in our previous work (Daher, Stewart *et al.* 2014). Twelve blades were tested and each initially contained RPA-exo lyophilized pellet. Ten blades had a sample solution containing 10^6 *Bacillus* spores and 10^6 copies of GBS genomic DNA. Two GBS negative control blades contained in the sample solution 10^6 *Bacillus* spores only. The RPA rehydration solution contained

equimolar amounts of primers and probes for both GBS and *B. atrophaeus*. The blades were then subjected to the operation sequences described previously (table 5.1). Fluorescence was acquired simultaneously in both FAM (GBS detection) and Texas Red® (*Bacillus* detection) channels.

5.3 Results

5.3.1 Valves obstruction

Under a BX-53 optical microscope (Olympus) (10X objective), it was observed that the150 µm nominal diameter glass beads of chamber (A) that were initially used for the lysis step, contained abnormally small (< 50 µm) beads that obstructed both capillary valves 1 and 2 (figure 5.3). Such obstruction was frequently observed causing microfluidic failure in some blades.

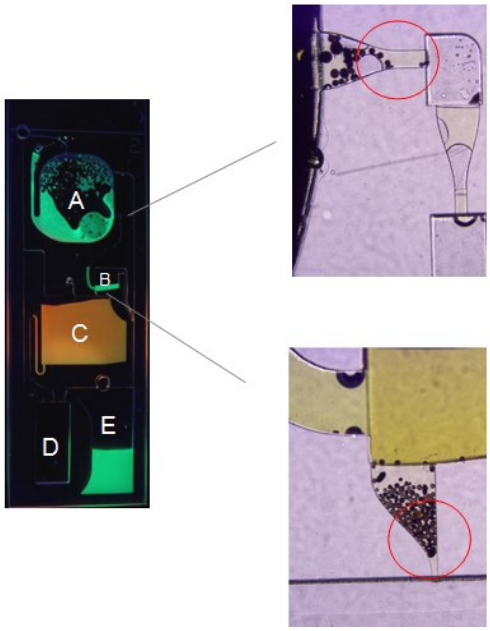

Figure 5.3. Obstruction of capillary valves by glass beads

On the left part is a picture of the blade taken under UV light showing retained fluids in different chambers at the end of operation sequences. The upper right picture is a microscopic magnification (10X objective) depicting the capillary valve 1 connecting chamber (A) to (B), obstructed by small-sized beads. The lower right picture is a microscopic magnification showing the capillary valve 2 connecting chamber (B) to (C) obstructed by small-sized beads.

5.3.2 GBS amplification simplex assays

For the batch of blades loaded with RPA basic reagent pellet, 6/8 (75%) of the blades gave positive amplification of GBS *cfb* target gene as shown by a band of 234 bp on 2% agarose gel electrophoresis (figure 5.4). One blade yielded no amplicon while another was dysfunctional due to valves obstruction as shown in figure 5.3. The last two blades were used as negative controls without genomic DNA and both yielded no amplicon as expected.

For the blades with RPA-exo reagent, 6/9 (67%) appeared fluidically functional. Out of the 6 functional blades, the negative control blade (blade N) did not yield amplification signal as expected, thus confirming the absence of contamination. The end point fluorescence signals intensity of the 5 other functional blades (n = 1-5) varied between 80 and 440 arbitrary fluorescence units (AFU) (figure 5.5). However, when compared to the negative control blade, only blades 4 and 5 produced exponential fluorescence curves. They were considered as strong positive for GBS target amplification as the intensities of their fluorescence signal was obtained in 10 min reaching a fluorescence intensity of 400 AFU. Blade 2 was considered as weak positive with a slow linear increase of fluorescence signal at 4 min reaching a fluorescence intensity of 180 AFU in 14 min forming a sigmoid amplification curve. Both blades 1 and 3 gave inconclusive results. Their respective curves were not exponential. A slight increase in fluorescence signal above the negative control signal (blade N) was observed after 10 min reaching a fluorescence intensity of 75 AFU and 125 AFU for blade 1 and 3, respectively. The remaining 3 blades (n = 7-9) were obstructed as described in section 5.3.1.

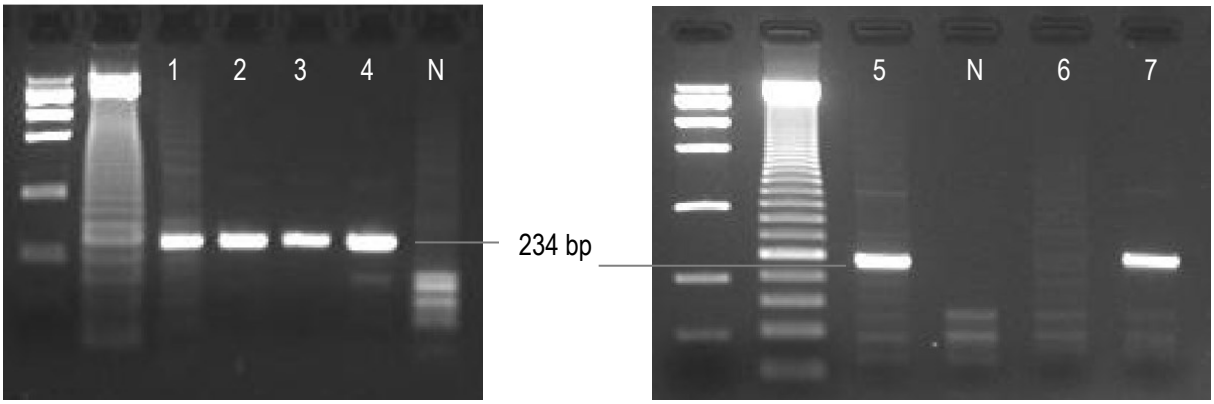

Figure 5.4. Amplification of GBS with the blade

Amplicons of expected size visualized on agarose gel electrophoresis with a band of 234 bp verified with a 1-kb molecular weight DNA ladder. Numbers 1 to 7 represent the number of tested blades; blade 6 gave a negative result. N= Non-template control.

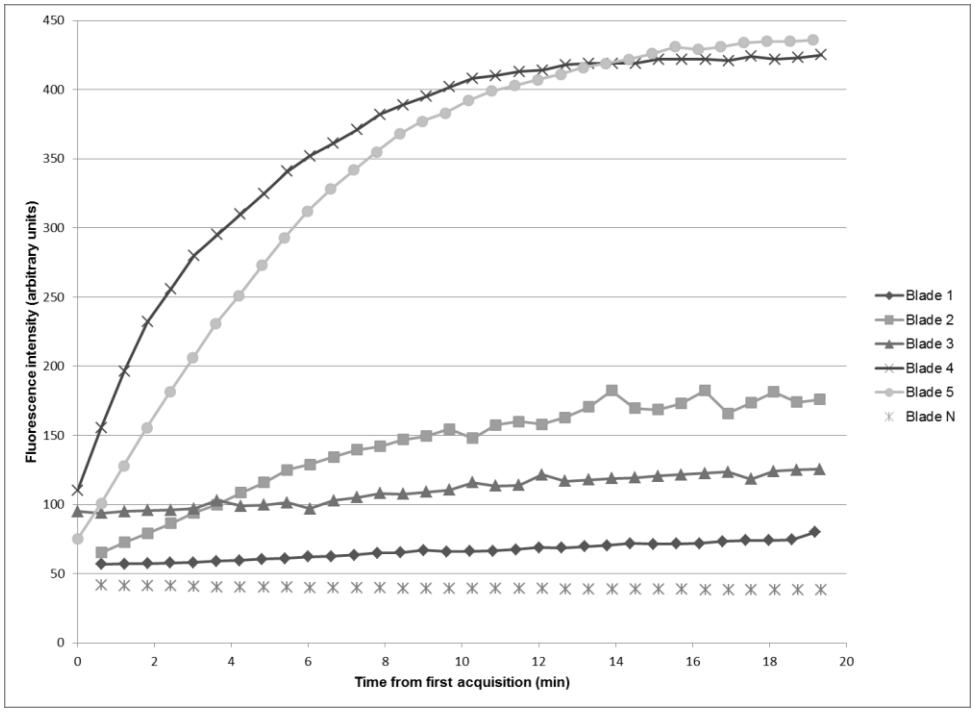

Figure 5.5. Fluorescence amplification signals of GBS with the blade

The graph depicts the time at which an initial amplification signal was observed and its increase in fluorescence intensity over time. Numbers in the legend 1 to 5 represent the number of tested blades that had functional microfluidic. N= Non-template control.

5.3.3 Sample-to-answer multiplex assays

All 12 blades were initially delivered by the NRC with moistened RPA-exo reagents and the reason was unknown. This had a negative effect on amplification yield: 5/8 (63%) of microfluidically functional blades gave an increase in fluorescence signals. As shown in figure 5.6, fluorescence signals in FAM channel for the detection of GBS were observed in all 5 blades and the intensity of the fluorescence signal varied between 80 and 350 AFU. Of these blades, only 4 blades yielded fluorescence signals in Texas® Red channel for the detection of *Bacillus* spores and the fluorescence intensity was relatively weak between 5 and 40 AFU. Three other blades (n= 9-11) were dysfunctional due to delamination of the elastomeric layer from the slide. The final blade (n=12) was a GBS negative control blade and did not give any fluorescence signal for *Bacillus* spores.

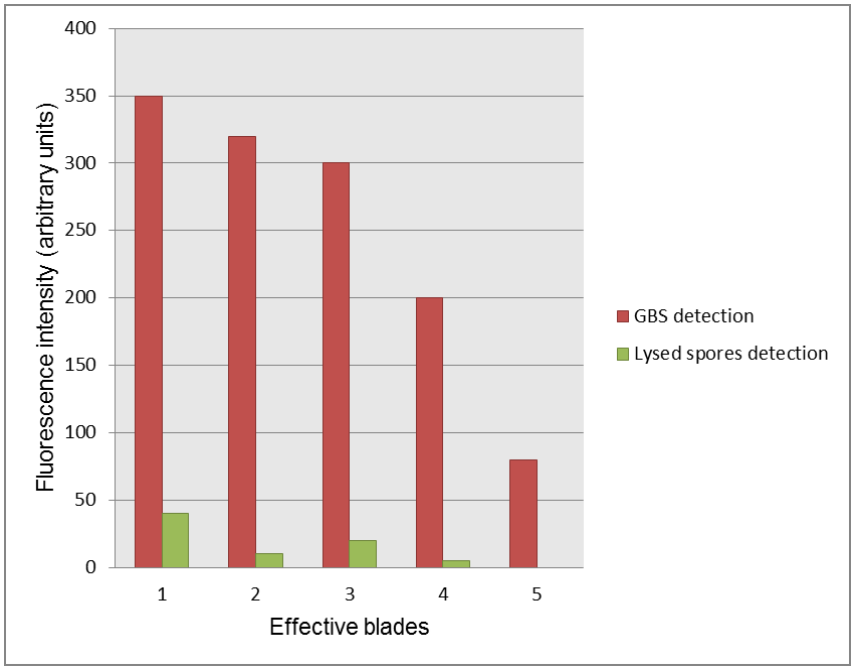

Figure 5.6. Multiplex assay results

Five of eight fluidically functional blades containing GBS DNA and *Bacillus* spores in sample solution gave fluorescence signals. Grey bars depict the fluorescence signal intensity of GBS and black bars correspond to the fluorescence signal intensity of *Bacillus* spores.

5.4 Discussion

The objective of this work which was to automate RPA assay from sample-to-answer, was just partially accomplished. Despite some failures that were encountered during this project, the blade and NADTech instrument succeeded to automate key functions in a sequential microfluidic process from sample preparation, target amplification, and detection (result generation whether positive or negative) in 19 blades.

With the blade, it was possible to perform an automated sample preparation in 7 min (table 5.1) without further hands-on manipulations. The blade sample preparation consisted of 3 steps: sample homogenization, mechanical lysis with glass beads, and heat deactivation of inhibitors. These steps usually take >1 hour for completion with standard bench-based sample preparation procedures. On the other hand, during the heat deactivation step, RPA reagents (*i.e.* rehydration solution and lyophilized pellet) will be subjected to an increase in temperature reaching 95°C for 7 min. Most thermolabile proteins/enzymes would be denatured at such elevated temperature however, the RPA lyophilised pellet showed tolerance and resistance to heat. This was demonstrated in an assay where RPA lyophilised pellets were heated at 95°C for different time interval (*i.e.* 1, 3, 5, and 10 min) in an oven and then tested for GBS amplification. We observed that all heat-treated lyophilised pellets performed as well as fresh pellets (Appendix 2, figure 5.7).

Target amplification using RPA reagents necessitated a modification to the RPA manufacturer protocol. According to manufacturer guidelines, magnesium ion should be added at the final stage just before incubation since it is the initiator of theRPA reaction (TwistDx 2015a). In the blade, we added magnesium to the rehydration solution that received subsequently 10µl of lysate. Hence, we demonstrated that magnesium could be added simultaneously with target nucleic acid in the rehydration solution without affecting RPA reaction efficiency. Our results demonstrated that an amplification of GBS took place. This was verified on agarose gel electrophoresis when integrating RPA basic reagents in the blade.

The results of the amplification indicated that the surface chemistry of the blade was not inhibitory to RPA composition. In addition, with NADTech instrument fluorescence signals in 2 channels were detected and illustrated as exponential curves on a computer screen. For the simplex assay and despite the low reproducibility of results (n=3, blades with potentially positive GBS amplification), GBS amplification was observed in 2-4 min for strong positive samples. On the other hand, with the multiplex assay using *Bacillus* spores simultaneously with GBS genomic DNA, the lysis step in the blade could not be confirmed. Weak fluorescence signals for *Bacillus* spores were observed compared to strong GBS fluorescence signals, leading thus to two assumptions: either the lysis process failed or that multiplexing was not successful in the blade due to targets competition. The negative control blades gave negative amplification results demonstrating that the blade system could be used for clinical assays without the risk of contamination. In addition, the blade and NADTech instrument gave short turnaround time estimated to 29 min for simplex assay and 44 min for multiplex assay (table 5.1). For RPA bench-based assays, the estimated turnaround time required to perform similar sample-to-answer tests (from sample preparation to results generation) is between 60-75 min depending on the number of samples to be tested. Hence, the blade system betters RPA bench-based tests in the total turnaround time which is due to the automation of cumbersome hands-on manipulations.

As a proof-of-concept, there are still some additional assays of optimization, verification and validation to perform on the actual prototype. The blade showed some microfluidic impairments (~ 23% of cases) that were reflected in some cases in bonding failure and in another cases in obstruction of capillary valves. Another noticeable problem was the integration of moistened enzymes in the blade which had deleterious effect on amplification yield. Moistened enzymes that were delivered from the NRC could be due to uncontrolled atmosphere conditions in working areas (high humidity) or to non-hermetic transport bags. Therefore, it is crucial to resolve such problems in order to have functional and efficient microfluidic cartridges. It is also important to have high reproducibility and

replicability with the blade in order to compare its performance with in-tube standard bench tests. This could be achieved by incorporating assay controls such as positive and negative controls that will help in establishing guidelines for interpretation of results. The importance of gaining robustness of the blade will have a direct impact on the 3 major operational steps: sample preparation, target amplification and signal acquisition. These steps are required for POCTs (Hartman, Ruiz *et al.* 2013).

The current NADTech instrument will be subjected to some adjustments. In order to monitor the performance of the blade and the NADTech instrument during an actual test, a special cover will be designed for attaching a camera, a strobe, and a tachometer in the instrument without altering its operating parameters. Picture captions will be taken showing fluid movement during the test and assembled in a video sequence using a Matlab® code. This will help to elucidate potential and future impairments either in the microfluidic functionality of the blade or in the centrifugation sequence of the instrument.

The optimization of the blade will offer a "mature" microfluidic cartridge ready for use in POC settings while giving rapid sample-to-answer results; estimating < $10 USD per blade per test. The final format for NADTech instrument will eventually offer a light, portable and low cost (< $500 USD) (de Paz, Brotons *et al.* 2014) machine that could be affordable for the developing world.

Chapter

VI. General Discussion & Perspectives

This doctoral project was focused on developing new strategies for target-specific nucleic acid amplification/detection. As mentioned previously in this thesis, despite the extensive use of PCR technology in molecular diagnostics, it is still very challenging for on-site applications especially in resource-poor settings as a result of the lack of infrastructure and trained personnel. Hence, our interest towards PCR alternatives such as isothermal amplification technologies arose since the latter generate billions of copies of a specific template starting with a single copy of the target. In addition, as their name indicates, some isothermal technologies (table 6.1) function at a single temperature without further temperature fluctuations which simplifies instrumentation.

6.1 Why evaluate recombinase polymerase amplification technology

At the beginning of this doctoral project, we made a general overview on the existing and emerging isothermal technologies. By comparing specific features of certain isothermal technologies (table 6.1), we were able to identify isothermal methods which showed promise to perform nucleic acid tests close to patient. These were helicase-dependent amplification (HDA), recombinase polymerase amplification (RPA), and ribonuclease-mediated amplification (RMA).

For the selection, specific criteria were taken into consideration: 1) run-time, 2) incubation temperature, 3) primers/probe design complexity, 4) detection methods, 5) multiplexing capability, 6) compatibility with miniaturized sample process, 7) commercially available reagents, and 8) capable of amplifying both DNA and RNA. The run-time reflects the speed of the isothermal technology in generating results. Ideally, the speed of reaction must be less than an hour. The incubation temperature is an important element since it is associated with the complexity of instrumentation required for isothermal amplification. In general, isothermal technologies functioning at lower temperatures (e.g. 30-42°C) necessitate less complex instrumentation; it is therefore advantageous to work especially with those that do not require an initial denaturation step at 95°C. For instance, among the listed isothermal technologies in table 6.1 only 5 out of 10 are truly isothermal. These techniques (HDA, RPA, NEAR, RMA, and CPA) operate nucleic acid amplification at one temperature throughout the

reaction alleviating the need for temperature fluctuation and control and thus simplifying instrumentation. However, most of the published works were using simple instruments to operate RPA such as ovens or heat blocks. Sodium sulfate was used as exothermic heat source for RPA reaction operation (Lillis, Lehman *et al.* 2014). This makes RPA suitable for non-instrumented nucleic acid amplification (NINA) platform as demonstrated recently with LAMP technology by work done by scientists from the PATH institute (LaBarre, Hawkins *et al.* 2011). Moreover, the capacity of RPA to function using only body heat was also demonstrated (*i.e.* a person's underarm) (Crannell, Rohrman *et al.* 2014a). To our knowledge, RPA was the first isothermal technology to demonstrate non-instrumentation requirements (body heat) which is very important criteria for POC applications. In addition, the stability of RPA due to the reagents dried format allows transportation and storage without refrigeration (at least up to one year (Erh-Chia Yeh and Lee 2013)). This means that a broad range of end users can operate RPA especially in low resource settings.

Furthermore, primers and probes design requirements play a key role in the specificity as well as the feasibility of multiplexing. Taking for example loop-mediated amplification (LAMP), 4-6 primers recognising 6 different regions in the target sequence are required rendering the amplification process highly specific yet multiplexing is more challenging due to the production of non-specific amplification products (de Paz, Brotons *et al.* 2014; Yan, Zhou *et al.* 2014). However, multiplexing with LAMP was made feasible by distinguishing different targets based on differences in primers melting temperature (Tm). Multiplex-LAMP assays have been developed to detect *stx*1 and *stx*2 simultaneously (Dong, Cho *et al.* 2014) as well as for the discrimination of influenza A subtypes H1 and H3, and influenza B (Mahony, Chong *et al.* 2013). On the other hand, other isothermal technologies require certain particularities for primers and probe design such as strand displacement amplification (SDA) and nicking enzyme amplification reaction (NEAR) that require short chimeric primers containing both DNA/RNA bases (Craw and Balachandran 2012) or long (30-35/46-52 bases) RPA primers/probes that are documented in the introduction of this thesis. Despite the requirement for long primers and probes for the RPA reaction, several multiplex assays (4 tube-based assays and 3 on-chip solid-phase assays) have been reported and discussed previously in the

introduction (Piepenburg, Williams *et al.* 2006; Crannell, Rohrman *et al.* 2014b; Daher, Stewart *et al.* 2014; Kersting, Rausch *et al.* 2014a; Santiago-Felipe, Tortajada-Genaro *et al.* 2014a; Santiago-Felipe, Tortajada-Genaro *et al.* 2015; Teoh, Sam *et al.* 2015).

Moreover, the detection strategies offered by isothermal technologies are associated with both the speed of analysis and the complexity of instrumentation; simple detection methods are usually fast. For instance, RPA technology offers several detection strategies either real-time or post-RPA amplification detection. RPA real-time detection is performed with fluorescent probes generating results in 5-15 min and these probes were detailed in chapter I section 1.4. For post-amplification detection methods, RPA uses agarose gel electrophoresis (~60 min for results), and lateral-flow strips (20-35 min) (chapter I, tables 1.2, 1.3A and 3B). For other methods such as LAMP, the application of probe-based detection methods was not feasible due to the cauliflower-like structures of LAMP products (Dong, Cho *et al.* 2014). Therefore, real-time LAMP detection is restricted to fluorescent dyes. Other post-amplification LAMP detection methods could be made by the naked eye as a result of turbidity due to pyrophosphate by-product formation (Notomi, Okayama *et al.* 2000). However, with end point analysis (gel electrophoresis, lateral flow or turbidimetry) as in case of HDA, NASBA or LAMP results are generated belatedly in 60-180 min depending on the assay (Craw and Balachandran 2012). Furthermore, LAMP products give a ladder-like pattern on an agarose gel whereas RPA purified-amplicons can be directly identified by a specific band on the agarose gel (Kersting, Rausch *et al.* 2014b).

Consequently, HDA and RMA (which is an improved form of HDA) were chosen for simplicity of their primer design (similar as those for PCR except that RMA primers contain a single RNA base linkage near a blocked 3'-end) and the reduced hands-on interventions (e.g. temperature/reagent additions). RPA was chosen for its speed of reaction (*i.e.* 20 min), the versatility of detection methods, and its simplified hands-on intervention. All three technologies are novel isothermal methods published in 2004 for HDA (Vincent, Xu *et al.* 2004), 2006 for RPA (Piepenburg, Williams *et al.* 2006), and around 2010 for RMA (Great Basin Corporation, Salt Lake, UT); therefore they were appealing for further investigations. HDA IsoAmp II&III Universal tHDA kits (Biohelix,

Beverly, MA), RPA Twistamp® basic kit (TwistDx, UK), and RMA manufacturer recipe (Great Basin Corporation, Salt Lake, UT) were tested in comparative assays to evaluate which of these isothermal technologies had the greatest potential in diagnostics. They were tested in a sensitivity assay for the amplification of plasmid DNA encoding for influenza A virus. In our hands, RPA suppressed HDA both in terms of speed of reaction (20 min versus 75 min) and sensitivity (10 copies versus 1000 copies of plasmid DNA). Unfortunately, RMA did not function at all despite several trials of performing it as recommended by Great Basin guidelines and even after they designed specific primer sequences for our assays.

Moreover, when we initiated this doctoral project in 2010, there were only a few papers of RPA technology; 3 published works (figure 1.1, chapter I). Hence, RPA evaluation in diagnostics became the main focus of this doctoral project.

Table 6.1. Short list of some isothermal amplification technologies

Technology[1]	Target	Initial denaturation[2]	Incubation (ºC)	Reaction time (min)	Multiplex	Primers required	Commercial test (Manufacturer)	FDA approved Tests[3]	Reference
HDA	DNA	N	65	30-120	Y	2	Y (Biohelix)	Y	(Vincent, Xu *et al.* 2004; Jeong, Park *et al.* 2009; Niemz, Ferguson *et al.* 2011; Deng and Gao 2015; U.S. Food and Drug Administration 2015)
RPA	DNA	N	37-42	20-40	Y	2	Y (TwistDx)	N	(Piepenburg, Williams *et al.* 2006; Lutz, Weber *et al.* 2010; Niemz, Ferguson *et al.* 2011; Deng and Gao 2015)
NASBA	RNA (DNA)	Y	41	60-180	Y	2	Y (Organon Teknika Corporation/bioMerieux)	Y	(Gracias and McKillip 2007; Niemz, Ferguson *et al.* 2011; Yan, Zhou *et al.* 2014; Deng and Gao 2015; U.S. Food and Drug Administration 2015)
TMA	RNA (DNA)	Y	42	60-180	Y	2	Y (Hologic/Gen-Probe)	Y	(Hofmann, Dries *et al.* 2005; Niemz, Ferguson *et al.* 2011; Deng and Gao 2015; U.S. Food and Drug Administration 2015)
LAMP	DNA	Y	60-65	60	Y	4-6	Y (Meridian Bioscience)	Y	(Curtis, Rudolph *et al.* 2009; Mori and Notomi 2009; Niemz, Ferguson *et al.* 2011; Deng and Gao 2015; U.S. Food and Drug Administration 2015)
RCA	DNA	Y	30-65	60-240	N/A	1	N/A	N	(Johne, Muller *et al.* 2009; Niemz, Ferguson *et al.* 2011; Deng and Gao 2015)
SDA	DNA	Y	30-55	60-120	Y	4	Y (Becton Dickinson/BD diagnostic systems)	Y	(Hellyer and Nadeau 2004; McHugh, Pope *et al.* 2004; Yan, Zhou *et al.* 2014; Deng and Gao 2015; U.S. Food and Drug Administration 2015)
NEAR	DNA	N	55-60	60-120	Y	2	Y (Alere)	Y	(Van Ness, Van Ness *et al.* 2003; Deng and Gao 2015)
RMA	DNA	N	65	60-90	Y	2	Y (Great Basin)	N	(Niemz, Ferguson *et al.* 2011; Great Basin Corporation 2015)
CPA	DNA	N	63-65	60	N/A	5-8	N/A	N	(Fang, Li *et al.* 2009; Niemz, Ferguson *et al.* 2011)

[1] HDA= Helicase-dependent amplification, RPA= Recombinase polymerase amplification, NASBA= Nucleic acid sequence-based amplification, TMA= Transcription-mediated amplification, LAMP= Loop-mediated amplification, RCA= Rolling circle amplification, SDA= Strand displacement amplification, NEAR= Nicking enzyme amplification reaction, RMA= ribonuclease-mediated amplification, and CPA= Cross priming amplification. [2] Isothermal technologies requiring initial denaturation of target nucleic acid at 95°C (N= No; Y=Yes; N/A= not applicable). [3] FDA approved tests for HDA= IsoAmp HSV Assay; NASBA= NucliSens CMV pp67, NucliSens EasyQ Enterovirus vl.1 Assay, and NucliSens EasyQ MRSA Assay; TMA= Aptima Combo 2 Assay, Aptima Combo 2 Assay (Panther System), and Gen-Probe AMPLIFIED *Chlamydia trachomatis* Assay Kit; LAMP= Illumigene *C. difficile* DNA amplification Assay, Illumigene *C. difficile* Assay, Illumigene Mycoplasma DNA Amplification Assay, Illumigene Group A Streptococcus (GAS) DNA Amplification Assay, and Illumigene Group B Streptococcus (GBS) DNA Amplification Assay; SDA= BD ProbeTec *Chlamydia trachomatis* (CT) Qx Amplified DNA Assay, BD ProbeTec ET *Chlamydia trachomatis* and *Neisseria gonorrhoeae* Amplified DNA Assays, BD ProbeTec Neisseria gonorrhoeae (GC) Qx Amplified DNA Assay, and BD ProbeTec Herpes Simplex viruses (HSV 1 & 2) Qx Amplified DNA Assays; NEAR= Alere I Influenza A&B assay (U.S. Food and Drug Administration 2015).

6.2 Retrospective analysis on thesis objectives and perspectives

6.2.1 RPA specificity and application in diagnostics

In order to assess the flexibility of RPA in various molecular diagnostic applications, it was important to understand the tolerance of RPA towards sequence mismatches. The challenge was in the assay design itself. It is known that RPA exo and nfo probes serve as forward primers after nuclease cleavage (chapter I, section 1.4). Second, RPA nuclease enzymes (*i.e.* exonuclease III and Nfo endonuclease) recognize automatically and cleave mismatched bases found in primers/probe sequences after their hybridization to target DNA (Piepenburg, Williams *et al.* 2014). Hence, it was obvious that RPA probe-based detection kits (*i.e.* Twistamp® exo, fpg and nfo kits) were not appropriate for our RPA specificity assays. Therefore, the RPA basic format was used to minimize results bias since no probes are used in this kit.

We proposed to study the specificity of RPA in discriminating closely related non-target sequences that bear naturally occurring mismatches; in the first place, for a common housekeeping gene *tuf*, and in the second place, for a species-specific gene *cfb* of Group G Streptococci. In addition, we assessed the effect of mismatch distribution on RPA reaction efficiency. As demonstrated in chapter III of this thesis, a total of 87 synthetically mismatched primer sequences representing 14 types of mismatches for 6 different bacterial sequences were tested. The results that were published in *Molecular and Cellular Probes* proposed additional considerations relative to the application of RPA in diagnostics. Besides manufacturer guidelines for RPA primers/probes design, we raised the fact that initial screening of RPA primers/probes alone will not ensure high specificity for RPA assay (Daher, Stewart *et al.* 2015). In fact, in order to gain specificity with RPA, we showed that target sequence regions for RPA amplification should have high sequence differences (heterology) to closely related non-target sequences. The percentage of heterology between target and non-target sequences is relative

to the amplicon size; we propose >36% of heterology with respect to the length of the amplified region (Daher, Stewart *et al.* 2015).

Although RPA can amplify long template sequences (up to 1.5 kb) (Piepenburg, Williams *et al.* 2006) yet RPA reaction kinetics is faster with shorter amplicons (*i.e.* 80-200 bp) (TwistDx 2015a). However, Li *et al.* raised the statement that RPA is best suited to long template amplification due to long RPA primer and probe requirements (Li and Macdonald 2015). To answer adequately to this statement, we should consider RPA enzymatic mechanism in other words, recombinases function. Knowing that the RPA amplification process is primarily dependent on recombinase with other key and accessory components, thus the speed of the amplification reaction will be dependent on recombinases. During RPA reaction, recombinases mediate homology search and strand exchange which is fueled by ATP-hydrolysis; 20-30 ATP/min per recombinase monomer and 3-10 bases exchanged/sec (Jain, Cox *et al.* 1994). This means that for long amplicons (>400 bp), recombinase proteins will need more ATP-hydrolysis and more time to complete their homology search and strand exchange mechanisms. This confirms the first statement that RPA kinetics will be faster for short amplicon sequences.

The challenge of developing an RPA assay for the screening of GBS was to perform RPA from clinical samples. We had also the concern to bring out the assay to achieve the desired speed of reaction (*i.e*. <1 hour) with high sensitivity and specificity. Hence, to gain speed and specificity, RPA exo probe (TwistAmp® exo kit) was used. As previously described, real-time exo probe generates results in 5-10 min and acts as forward primer after nuclease cleavage increasing thus the specificity (section 1.4, chapter I). As described in chapter IV, the proposed RPA-GBS assay was fast (time-to-result <20 min), sensitive (96% clinically), and specific (100% clinically). This assay demonstrated once again the feasibility of multiplexing with RPA and the assay included a process control. We were the first to test RPA with human clinical samples; vaginal/anal samples were used for the screening of GBS. It is noteworthy that during this doctoral project RPA was evaluated for inhibitory effect with different types of clinical samples including

nasal/pharyngeal swabs and stool samples. Surprisingly, RPA showed a good tolerance to inhibitors in both types of samples (Appendixes 1 & 3, table 1.4 & figure 6.1, respectively). Hence, we assume that with appropriate sample preparation direct RPA amplification and detection may be feasible in crude samples. For instance, by consolidating sample preparation with nucleic acid amplification/detection in an automated system, Erh-Chia and colleagues were able to detect MRSA DNA directly from spiked blood sample. The process consisted of inertial plasma separation avoiding red blood cells lysis and thereby eliminating a potential PCR-inhibitor, hemoglobin. This system proved to have >99% separation efficiency (Erh-Chia Yeh and Lee 2013). Ultimately, the best sample preparation approach should be adjusted for each type of application and should consider many factors such as pathogen concentration, presence of inhibitors and lysis efficiency.

On the other hand, the incorporation of more resistant enzymes/proteins in RPA reagents could save from sample preparation step. For instance, a novel OmniAmp DNA polymerase derived originally from PyroPhage 3173 DNA polymerase was used in a LAMP assay for the detection of RNA and DNA targets (Chander, Koelbl *et al.* 2014). While being thermostable, OmniAmp polymerase features an inherent reverse transcriptase activity with efficient strand-displacement activity amplifying RNA, DNA, and cDNA templates. The latter showed higher kinetics than *Bst* polymerase (time-to-result < 30 min) and simple heat lysis step was sufficient to offer good tolerance to whole blood inhibitors. This enzyme is commercialized by Lucigen (Lucigen Corporation, Middleton, WI) and is compatible with dry format with up to 6 months stability at room temperature (Chander, Koelbl *et al.* 2014). All these features make OmniAmp polymerase interesting for RPA formulation. However, the optimal temperature of this enzyme is 72°C which is beyond the range of RPA (25-42°C). On the other hand, thermostable RecA proteins (Tth RecA) as well as thermostable SSB (ET SSB) are available at New England Biolabs (New England Biolabs Ltd., Whitby, ON). This makes the formulation of new thermostable RPA with the incorporation of OmniAmp polymerase very interesting and challenging at the same time. This combination could offer more

resistant RPA reagents that do not require any purification or treatment of clinical samples and a robust one step RT-RPA for RNA pathogens.

6.2.2 RPA automation and perspectives

The last objective of this thesis was to automate the RPA assay by its integration into microfluidic cartridge for POC applications. Miniaturized into a microfluidic nucleic acid amplification tests offer many advantages in diagnostics: 1) reducing hands-on manipulations, 2) short turnaround time, 3) reduced costs, and4) portable devices moving the assay near the patient (Bell and Selvarangan 2014). Reducing hands-on manipulations implies small reaction volumes and simple inexpensive instrumentation. On the other hand, shortening turnaround time involves fast and accurate sample preparation, target nucleic acid amplification, and detection. Finally, making the diagnostic assay near the patient involves the development of a portable assay that could be performed with untrained personnel.

Owing to RPA fast reaction speed of RPA (<20 min), real-time detection with exo probe, and freeze dried reagent formulation, it seemed possible to integrate RPA into lab-on-chip platform. Our objective was to generate a microfluidic device named blade consisting of an automated sample-in/answer-out system. As described in chapter V, the blade is a small plastic slide embossed with a layer of soft plastic grafted with separate biological chambers interconnected via micro-channels (Chapter V, figure 5.1). All biological steps from sample treatment to target detection were controlled by programmed centrifugal sequences of an electrically powered instrument accommodating 2 blades at the same time (Chapter V, figure 5.2). While conducting this objective, numerous challenges were faced. First, the conception of the blade and the instrument implicated the choice of appropriate materials and later their fabrication. Second, we defined conditions for the incorporation of lysis solution and RPA reagents in the blade while maintaining their functionality. Finally, setting RPA protocol in the blade involved the identification of sample volumes for each reaction chamber, reaction temperature,

microfluidic sequence operation, and assay run time. Consequently, we adopted a different RPA protocol from the manufacturer's recommendations. We demonstrated that magnesium acetate (the initiator for RPA reaction) that is usually added at the final step; could be added simultaneously with target nucleic acid in the buffer solution before reconstituting the RPA lyophilized pellet. Despite the numerous RPA applications, few works have included an on-chip sample preparation (Kim, Park *et al.* 2014; Ming, Kim *et al.* 2015). Our blade system is among the first to combine an automated on-chip sample preparation (extraction, lysis, and denaturation of contaminants) necessitating as few as 7 min for completion. Our preliminary results demonstrated that the blade system was able to generate a sample-to-answer result in 44 min. However, as many proof-of-concept assays, it takes a relatively long time for improving and validating the platform before achieving the final version. Nonetheless, the blade with RPA technology offers high flexibility working with both DNA and/or RNA targets. The actual blade was tested with RNA transcript of Influenza A virus but the results were not presented due to low reproducibility (data not shown). Our next challenge will be of making the platform fully automated with only a single sample loading step and capable of treating a wide range of clinical samples while offering results in less than an hour.

Other interesting low cost microfluidic platforms have also been developed and should be compared with the blade system. These platforms combine nucleic acid extraction with isothermal nucleic acid amplification/detection with minimal or no-electrical power source (Bell and Selvarangan 2014; Linnes, Fan *et al.* 2014). HDA isothermal amplification has been performed using low cost materials such as paper, pipette tips, or microfluidic polymer chip. Simple toe warmers served as exothermal heat source in a Styrofoam cup. The toe warmers insured optimal HDA temperature of 65°C for 55 min. Different extraction and sample preparation methods were described. Huang, Do *et al.* have extracted *Clostridium difficile* DNA in stool samples of infected patients using an air driven polymer solid-phase extraction method. The latter consisted of a three-step alcohol precipitation using a chaotropic agent in which lysed DNA was captured by glycogen particles (Byrnes,

Fan *et al.* 2013). On the other hand, Linnes, Fan et *al.* have demonstrated the extraction of *Chlamydia trachomatis* DNA in synthetic urine with similar solid-phase extraction in a paper tip support. Compared to the polymer solid-phase extraction method, the paper-based extraction and amplification had faster assembly (<1% of the time required for polymer solid-phase extraction) but slower flow rate (Linnes, Fan *et al.* 2014). Since, the paper-based extraction protocol does not require any heat treatment and the presence of glycogen particles enhances DNA capture, it will be interesting to combine paper-based extraction with RPA amplification in an instrument-free assay especially that RPA could be performed at body temperature (Crannell, Rohrman *et al.* 2014a). This combination could offer portable, inexpensive and sample-to answer platform functioning at body temperature amenable for POC applications.

Nevertheless, bringing RPA into POC applications requires also simple instrument-free detection methods that can be performed by unqualified personnel. It has been demonstrated by Tanner and colleagues that the amplicon formation can be monitored by visual detection of pH change using pH-sensitive dyes (Bell, Bonner *et al.* 2014). The study is based on the fact that polymerase enzyme generates pyrophosphate and hydrogen ions during DNA synthesis. Hence, the pH of the amplification reaction will drop by the end of the PCR cycle due to the accumulation of hydrogen ions. They used pH-sensitive dyes to monitor LAMP amplification reaction in alkaline buffer (pH 8.8-9.0) and demonstrated that a visual color change was observed after 15 min at 65°C of amplification in template positive samples indicative of proton accumulation (Bell, Bonner *et al.* 2014). The application of pH-sensitive dyes for the visual detection of RPA positive amplification has not been demonstrated yet. Despite the viscosity of RPA rehydration buffer the latter has an alkaline pH of 8.5 which makes it suitable for such analysis. On the other hand, the interference of inhibitory substances or clinical sample contaminants remains to be evaluated.

Another interesting method for visual detection of RPA amplicons was developed recently based on the flocculation principle. Briefly, flocculation is based on the

generation of long amplicons (>100 bp) that will precipitate onto a bead surface in certain salt buffer conditions. Than after a wash step, a low acetate pH buffer of 4.4 is added stimulating DNA loaded beads to flocculate changing the solution from cloudy to clear. Even though the extraction and amplification assays were not combined with the flocculation step, yet this detection method was proven to be efficient on a variety of samples including different plant pathogens and human diseases with a turnaround time <90 min (Wee, Lau *et al.* 2015).

Furthermore, RPA flexibility has been demonstrated with Smartphone technology. For instance, Ming and colleagues recently fused quantum dot barcode technology and RPA in a low-cost chip using Smartphone camera for a wireless detection of multiple pathogens simultaneously. The concept of pathogen detection with quantum dot barcode was analytically validated for the simultaneous detection of HIV and Hepatitis B/C in patient samples. This prototype is intended to perform all biological steps: DNA extraction, amplification and detection. The envisioned final device measuring 100 millimeters will be an enclosed system incorporating all reagents for sample preparation, RPA amplification, on-chip barcode hybridization, Smartphone imaging, and sample analysis. Fluid motion inside the device from one compartment to another would be electrically driven (Ming, Kim *et al.* 2015). It was also reported that scientists of the German Primate Center in collaboration with TwistDx have succeeded to develop a portable solar-powered, mobile, suitcase-sized laboratory for the rapid diagnosis of Ebola virus that was lately tested in Africa in the field. Another report has stated that RPA was actually tested in field for the detection of Foot-and-Mouth disease virus using 96 swabs from suspected cattle at quarantine stations in Hurghada and Safaga in Egypt (TwistDx 2015b).

At the end of this discussion, we note that RPA technology owes its high speed of reaction and its true isothermal profile to its "protein soup" that mimics the actual *in vivo* recombination process. Its low operation temperature (near room temperature) will eventually bring RPA reaction to be performed in someone’s hand with visual observation of results in less than 30 minutes. In the near future, RPA speed will probably be used for Next-Generation Sequencing (NGS) applications especially

that pre-PCR amplification are still required for generating standard libraries. By replacing PCR with RPA for library generation, the time required for PCR cycles will be shortened. It was demonstrated that RPA-amplified libraries had a reduced bias towards GC-rich templates and a low number of errors in base incorporation at low complexity regions (Oyola, Otto *et al.* 2012). This makes RPA amenable for NGS applications since that simple or non-instrumentation was demonstrated with RPA technology. Consequently, fusing RPA with NGS will have direct impact on diagnosis of pathogens by decreasing: 1) the cost for DNA sequencing, 2) turnaround time (from days to few hours (ideally 1 hour), and 3) making portable NGS platforms for on field tests.

Bibliography

Abd El Wahed, A., A. El-Deeb, M. El-Tholoth, H. Abd El Kader, A. Ahmed, S. Hassan, B. Hoffmann, B. Haas, M. A. Shalaby, F. T. Hufert and M. Weidmann (2013). "A portable reverse transcription recombinase polymerase amplification assay for rapid detection of foot-and-mouth disease virus." PLoS ONE 8(8): e71642 DOI: journal.pone.0071642.

Abd El Wahed, A., P. Patel, D. Heidenreich, F. T. Hufert and M. Weidmann (2013). "Reverse transcription recombinase polymerase amplification assay for the detection of middle East respiratory syndrome coronavirus." PLoS Curr 5 DOI: currents.outbreaks.62df1c7c75ffc96cd59034531e2e8364.

Aebischer, A., K. Wernike, B. Hoffmann and M. Beer (2014). "Rapid genome detection of Schmallenberg virus and bovine viral diarrhea virus by use of isothermal amplification methods and high-speed real-time reverse transcriptase PCR." J Clin Microbiol 52(6): 1883-1892 DOI: JCM.00167-14.

Ahmed, A., H. van der Linden and R. A. Hartskeerl (2014). "Development of a recombinase polymerase amplification assay for the detection of pathogenic Leptospira." Int J Environ Res Public Health 11(5): 4953-4964 DOI: ijerph110504953.

Álvarez-Fernández, R. (2013). Chapter One - Explanatory Chapter: PCR Primer Design. Methods in Enzymology. L. Jon, Academic Press. Volume 529**:** 1-21.

Amer, H. M., A. Abd El Wahed, M. A. Shalaby, F. N. Almajhdi, F. T. Hufert and M. Weidmann (2013). "A new approach for diagnosis of bovine coronavirus using a reverse transcription recombinase polymerase amplification assay." J Virol Methods 193(2): 337-340 DOI: j.jviromet.2013.06.027.

Asiello, P. J. and A. J. Baeumner (2011). "Miniaturized isothermal nucleic acid amplification, a review." Lab Chip 11(8): 1420-1430.

Bearinger, J. P. and L. C. Dugan (2011). "Methods for Point-Of-Care detection of nucleic acid in a sample." http://www.google.com/patents/US20110294112.

Bej, A. K., M. H. Mahbubani and R. M. Atlas (1991). "Amplification of nucleic acids by polymerase chain reaction (PCR) and other methods and their applications." Crit Rev Biochem Mol Biol 26(3-4): 301-334.

Bell, J., A. Bonner, D. M. Cohen, R. Birkhahn, R. Yogev, W. Triner, J. Cohen, E. Palavecino and R. Selvarangan (2014). "Multicenter clinical evaluation of the novel Alere i Influenza A&B isothermal nucleic acid amplification test." J Clin Virol 61(1): 81-86 DOI: j.jcv.2014.06.001.

Bell, J. J. and R. Selvarangan (2014). "Evaluation of the Alere I influenza A&B nucleic acid amplification test by use of respiratory specimens collected in viral transport medium." J Clin Microbiol 52(11): 3992-3995 DOI: JCM.01639-14

Bissonnette, L., S. Chapdelaine, R. Peytavi, A. Huletsky, G. Stewart, M. Boissinot, P. Allibert and M. G. Bergeron (2015). Global Point of Care: Strategies for disasters, emergencies, and Public Health Resilience. Washington, DC, AACC Press235-247.

Boyer, K. M. and S. P. Gotoff (1986). "Prevention of early-onset neonatal group B streptococcal disease with selective intrapartum chemoprophylaxis." N Engl J Med 314(26): 1665-1669 DOI: NEJM198606263142603.

Boyle, B., N. Dallaire and J. MacKay (2009). "Evaluation of the impact of single nucleotide polymorphisms and primer mismatches on quantitative PCR." BMC Biotechnol 9: 75 DOI: 10.1186/1472-6750-9-75.

Boyle, D. S., D. A. Lehman, L. Lillis, D. Peterson, M. Singhal, N. Armes, M. Parker, O. Piepenburg and J. Overbaugh (2013). "Rapid detection of HIV-1 proviral DNA for early infant diagnosis using recombinase polymerase amplification." MBio 4(2) DOI: mBio.00135-13.

Boyle, D. S., R. McNerney, H. Teng Low, B. T. Leader, A. C. Perez-Osorio, J. C. Meyer, D. M. O'Sullivan, D. G. Brooks, O. Piepenburg and M. S. Forrest (2014). "Rapid Detection of Mycobacterium tuberculosis by Recombinase Polymerase Amplification." PLoS ONE 9(8): e103091 DOI: journal.pone.0103091.

Byrnes, S., A. Fan, J. Trueb, F. Jareczek, M. Mazzochette, A. Sharon, A. F. Sauer-Budge and C. M. Klapperich (2013). "A portable, pressure driven, room temperature nucleic acid extraction and storage system for point of care molecular diagnostics." Analytical Methods 5(13): 3177-3184 DOI: c3ay40162f.

Chander, Y., J. Koelbl, J. Puckett, M. J. Moser, A. J. Klingele, M. R. Liles, A. Carrias, D. A. Mead and T. W. Schoenfeld (2014). "A novel thermostable polymerase for RNA and DNA loop-mediated isothermal amplification (LAMP)." Frontiers in Microbiology 5: 395 DOI: fmicb.2014.00395.

Chen, Z., H. Yang and N. P. Pavletich (2008). "Mechanism of homologous recombination from the RecA-ssDNA/dsDNA structures." Nature 453(7194): 489-484 DOI: nature06971.

Crannell, Z. A., M. M. Cabada, A. Castellanos-Gonzalez, A. Irani, A. C. White and R. Richards-Kortum (2014). "Recombinase Polymerase Amplification-Based Assay to Diagnose Giardia in Stool Samples." Am J Trop Med Hyg DOI: ajtmh.14-0593.

Crannell, Z. A., A. Castellanos-Gonzalez, A. Irani, B. Rohrman, A. C. White and R. Richards-Kortum (2014). "Nucleic acid test to diagnose cryptosporidiosis: lab assessment in animal and patient specimens." Anal Chem 86(5): 2565-2571 DOI: ac403750z.

Crannell, Z. A., B. Rohrman and R. Richards-Kortum (2014a). "Equipment-free incubation of recombinase polymerase amplification reactions using body heat." PLoS One 9(11): e112146 DOI: journal.pone.0112146

Crannell, Z. A., B. Rohrman and R. Richards-Kortum (2014b). "Quantification of HIV-1 DNA using real-time recombinase polymerase amplification." Anal Chem 86(12): 5615-5619 DOI: ac5011298.

Craw, P. and W. Balachandran (2012). "Isothermal nucleic acid amplification technologies for point-of-care diagnostics: a critical review." Lab on a Chip 12(14): 2469-2486 DOI: C2lc40100b.

Curtis, K. A., D. L. Rudolph and S. M. Owen (2009). "Sequence-specific detection method for reverse transcription, loop-mediated isothermal amplification of HIV-1." J Med Virol 81(6): 966-972 DOI: jmv.21490.

Daher, R. K., G. Stewart, M. Boissinot and M. G. Bergeron (2014). "Isothermal Recombinase Polymerase Amplification Assay Applied to the Detection of Group B Streptococci in Vaginal/Anal Samples." Clin Chem 60(4): 660-666 DOI: clinchem.2013.213504.

Daher, R. K., G. Stewart, M. Boissinot, D. K. Boudreau and M. G. Bergeron (2015). "Influence of sequence mismatches on the specificity of recombinase polymerase amplification technology." Mol Cell Probes 29(2): 116-121 DOI: j.mcp.2014.11.005.

Daniels, J., J. Gray, H. Pattison, T. Roberts, E. Edwards, P. Milner, L. Spicer, E. King, R. K. Hills, R. Gray, L. Buckley, L. Magill, N. Elliman, B. Kaambwa, S. Bryan, R. Howard, P. Thompson and K. S. Khan (2009). "Rapid testing for group B streptococcus during labour: a test accuracy study with evaluation of acceptability and cost-effectiveness." Health Technol Assess 13(42): 1-154, iii-iv.

Davies, H. D., M. A. Miller, S. Faro, D. Gregson, S. C. Kehl and J. A. Jordan (2004). "Multicenter study of a rapid molecular-based assay for the diagnosis of group B Streptococcus colonization in pregnant women." Clinical Infectious Diseases 39(8): 1129-1135 DOI: 10.1086/424518.

de-Paris, F., A. B. M. P. Machado, T. C. Gheno, B. M. Ascoli, K. R. P. de Oliveira and A. L. Barth (2011). "Group B *Streptococcus* detection: comparison of PCR assay and culture as a screening method for pregnant women." Brazilian Journal of Infectious Diseases 15(4): 323-327.

de Paz, H. D., P. Brotons and C. Munoz-Almagro (2014). "Molecular isothermal techniques for combating infectious diseases: towards low-cost point-of-care diagnostics." Expert Rev Mol Diagn 14(7): 827-843.

del Río, J. S., N. Yehia Adly, J. L. Acero-Sánchez, O. Y. F. Henry and C. K. O'Sullivan (2014). "Electrochemical detection of Francisella tularensis genomic DNA using solid-phase recombinase polymerase amplification." Biosensors and Bioelectronics 54(0): 674-678.

Deng, H. and Z. Gao (2015). "Bioanalytical applications of isothermal nucleic acid amplification techniques." Anal Chim Acta 853: 30-45 DOI: j.aca.2014.09.037.

Dong, H.-J., A.-R. Cho, T.-W. Hahn and S. Cho (2014). "Development of a multiplex loop-mediated isothermal amplification assay to detect shiga toxin-producing Escherichia coli in cattle." Journal of Veterinary Science 15(2): 317-325 DOI: jvs.2014.15.2.317.

Edwards, R. K., S. M. Novak-Weekley, P. P. Koty, T. Davis, L. J. Leeds and J. A. Jordan (2008). "Rapid group B streptococci screening using a real-time polymerase chain reaction assay." Obstetrics and Gynecology 111(6): 1335-1341.

Erh-Chia Yeh and L. P. Lee (2013). One-step digital plasma separation for molecular diagnostics. 17th International Conference on Miniaturized Systems for Chemistry and Life Sciences. Freiburg, Germany, μTAS. CBMS-0001**:** 1323-1325.

Escadafal, C., O. Faye, A. A. Sall, M. Weidmann, O. Strohmeier, F. von Stetten, J. Drexler, M. Eberhard, M. Niedrig and P. Patel (2014). "Rapid molecular assays for the detection of yellow fever virus in low-resource settings." PLoS Negl Trop Dis 8(3): e2730 DOI: journal.pntd.0002730.

Escadafal, C., J. T. Paweska, A. Grobbelaar, C. le Roux, M. Bouloy, P. Patel, A. Teichmann, O. Donoso-Mantke and M. Niedrig (2013). "International external quality assessment of molecular detection of Rift Valley fever virus." PLoS Negl Trop Dis 7(5): e2244 DOI: journal.pntd.0002244.

Euler, M., Y. Wang, D. Heidenreich, P. Patel, O. Strohmeier, S. Hakenberg, M. Niedrig, F. T. Hufert and M. Weidmann (2013). "Development of a panel of Recombinase Polymerase Amplification assays for the detection of biothreat agents." J Clin Microbiol 51: 1110-1117 DOI: JCM.02704-12.

Euler, M., Y. J. Wang, O. Nentwich, O. Piepenburg, F. T. Hufert and M. Weidmann (2012a). "Recombinase polymerase amplification assay for rapid detection of Rift Valley fever virus." Journal of Clinical Virology 54(4): 308-312 DOI: j.jcv.2012.05.006.

Euler, M., Y. J. Wang, P. Otto, H. Tomaso, R. Escudero, P. Anda, F. T. Hufert and M. Weidmann (2012b). "Recombinase Polymerase Amplification Assay for Rapid Detection of Francisella tularensis." Journal of Clinical Microbiology 50(7): 2234-2238 DOI: Jcm.06504-11.

Fang, R., X. Li, L. Hu, Q. You, J. Li, J. Wu, P. Xu, H. Zhong, Y. Luo, J. Mei and Q. Gao (2009). "Cross-priming amplification for rapid detection of Mycobacterium tuberculosis in sputum specimens." J Clin Microbiol 47(3): 845-847 DOI: JCM.01528-08

Gavino, M. and E. Wang (2007). "A comparison of a new rapid real-time polymerase chain reaction system to traditional culture in determining group B streptococcus colonization." Am J Obstet Gynecol 197(4): 388 e381-384.

Gill, P., M. Amini, A. Ghaemi, L. Shokouhizadeh, H. Abdul-Tehrani, A. Karami and A. Gilak (2007). "Detection of Helicobacter pylori by enzyme-linked immunosorbent assay of thermophilic helicase-dependent isothermal DNA amplification." Diagn Microbiol Infect Dis 59(3): 243-249 DOI: j.diagmicrobio.2007.05.005.

Gill, P. and A. Ghaemi (2008). "Nucleic acid isothermal amplification technologies - A review." Nucleosides Nucleotides & Nucleic Acids 27(3): 224-243.

Govindarajan, A. V., S. Ramachandran, G. D. Vigil, P. Yager and K. F. Bohringer (2012). "A low cost point-of-care viscous sample preparation device for molecular diagnosis in the developing world; an example of microfluidic origami." Lab Chip 12(1): 174-181.

Gracias, K. S. and J. L. McKillip (2007). "NUCLEIC ACID SEQUENCE-BASED AMPLIFICATION (NASBA) IN MOLECULAR BACTERIOLOGY: A PROCEDURAL GUIDE." Journal of Rapid Methods & Automation in Microbiology 15(3): 295-309.

Gray, J. W., P. J. Milner, E. H. Edwards, J. P. Daniels and K. S. Khan (2012). "Feasibility of using microbiology diagnostic tests of moderate or high complexity at the point-of-care in a delivery suite." Journal of Obstetrics and Gynaecology 32(5): 458-460.

Great Basin Corporation. (2015). "Isothermal amplification." Retrieved 15-04-2015, 2015, from http://www.gbscience.com/technology/iso-amp/.

Hakenberg, S., M. Hugle, M. Weidmann, F. Hufert, G. Dame and G. A. Urban (2012). "A phaseguided passive batch microfluidic mixing chamber for isothermal amplification." Lab Chip 12(21): 4576-4580.

Hartman, M. R., R. C. Ruiz, S. Hamada, C. Xu, K. G. Yancey, Y. Yu, W. Han and D. Luo (2013). "Point-of-care nucleic acid detection using nanotechnology." Nanoscale 5(21): 10141-10154.

Hattersley, S. M., J. Greenman and S. J. Haswell (2013). Methods in Molecular Biology. The application of microfluidic devices for viral diagnosis in developing countries. G. J. a. C. D. Mansfield, Springer Science+business Media. 949**:** 285-303.

Hellyer, T. J. and J. G. Nadeau (2004). "Strand displacement amplification: a versatile tool for molecular diagnostics." Expert Rev Mol Diagn 4(2): 251-261 DOI: ERM040213

Hill-Cawthorne, G. A., L. O. Hudson, M. F. El Ghany, O. Piepenburg, M. Nair, A. Dodgson, M. S. Forrest, T. G. Clark and A. Pain (2014). "Recombinations in Staphylococcal Cassette Chromosome mec Elements Compromise the Molecular Detection of Methicillin Resistance in Staphylococcus aureus." PLoS ONE 9(6): e101419 DOI: journal.pone.0101419.

Hofmann, W. P., V. Dries, E. Herrmann, B. Gartner, S. Zeuzem and C. Sarrazin (2005). "Comparison of transcription mediated amplification (TMA) and reverse transcription polymerase chain reaction (RT-PCR) for detection of hepatitis C virus RNA in liver tissue." J Clin Virol 32(4): 289-293 DOI: j.jcv.2004.08.011.

Huy, N. T., T. T. Hang le, D. Boamah, N. T. Lan, P. Van Thanh, K. Watanabe, V. T. Huong, M. Kikuchi, K. Ariyoshi, K. Morita and K. Hirayama (2012). "Development of a single-tube loop-mediated isothermal amplification assay for detection of four pathogens of bacterial meningitis." FEMS Microbiol Lett 337(1): 25-30.

Jain, S. K., M. M. Cox and R. B. Inman (1994). "On the role of ATP hydrolysis in RecA protein-mediated DNA strand exchange. III. Unidirectional branch migration and extensive hybrid DNA formation." J Biol Chem 269(32): 20653-20661.

Jaroenram, W. and L. Owens (2014). "Recombinase polymerase amplification combined with a lateral flow dipstick for discriminating between infectious Penaeus stylirostris densovirus and virus-related sequences in shrimp genome." J Virol Methods 208: 144-151 DOI: j.jviromet.2014.08.006.

Jeong, Y. J., K. Park and D. E. Kim (2009). "Isothermal DNA amplification in vitro: the helicase-dependent amplification system." Cell Mol Life Sci 66(20): 3325-3336.

Johne, R., H. Muller, A. Rector, M. van Ranst and H. Stevens (2009). "Rolling-circle amplification of viral DNA genomes using phi29 polymerase." Trends Microbiol 17(5): 205-211 DOI: j.tim.2009.02.004.

Jordan, J. A., G. Hall and T. Davis (2010). "Multicenter Study Evaluating Performance of the Smart Group B Streptococcus (GBS) Assay Using an Enrichment Protocol for Detecting GBS Colonization in Patients in the Antepartum Period." Journal of Clinical Microbiology 48(9): 3193-3197 DOI: Jcm.00106-10.

Ke, D., C. Menard, F. J. Picard, M. Boissinot, M. Ouellette, P. H. Roy and M. G. Bergeron (2000). "Development of conventional and real-time PCR assays for the rapid detection of group B streptococci." Clin Chem 46(3): 324-331.

Kersting, S., V. Rausch, F. Bier and M. von Nickisch-Rosenegk (2014a). "Multiplex isothermal solid-phase recombinase polymerase amplification for the specific and fast DNA-based detection of three bacterial pathogens." Microchimica Acta: 1-9.

Kersting, S., V. Rausch, F. F. Bier and M. von Nickisch-Rosenegk (2014b). "Rapid detection of Plasmodium falciparum with isothermal recombinase polymerase amplification and lateral flow analysis." Malar J 13: 99 DOI: 10.1186/1475-2875-13-99.

Kim, T. H., J. Park, C. J. Kim and Y. K. Cho (2014). "Fully Integrated Lab-on-a-Disc for Nucleic Acid Analysis of Food-Borne Pathogens." Analytical Chemistry 86(8): 3841-3848 DOI: ac403971h.

Krolov, K., J. Frolova, O. Tudoran, J. Suhorutsenko, T. Lehto, H. Sibul, I. Mager, M. Laanpere, I. Tulp and U. Langel (2014). "Sensitive and rapid detection of Chlamydia trachomatis by recombinase polymerase amplification directly from urine samples." J Mol Diagn 16(1): 127-135 DOI: j.jmoldx.2013.08.003.

LaBarre, P., K. R. Hawkins, J. Gerlach, J. Wilmoth, A. Beddoe, J. Singleton, D. Boyle and B. Weigl (2011). "A Simple, Inexpensive Device for Nucleic Acid Amplification without Electricity—Toward Instrument-Free Molecular Diagnostics in Low-Resource Settings." PLoS One 6(5): e19738 DOI: journal.pone.0019738.

Lee, T. Y., Y. Shin and M. K. Park (2014). "A simple, low-cost, and rapid device for a DNA methylation-specific amplification/detection system using a flexible plastic and silicon complex." Lab Chip 14(21): 4220-4229 DOI: c4lc00804a.

Li, J. and J. Macdonald (2015a). "Advances in isothermal amplification: novel strategies inspired by biological processes." Biosens Bioelectron 64: 196-211 DOI: j.bios.2014.08.069.

Li, J. and J. Macdonald (2015b). "Advances in isothermal amplification: novel strategies inspired by biological processes." Biosensors and Bioelectronics 64(0): 196-211 DOI: http://dx.doi.org/10.1016/j.bios.2014.08.069.

Lillis, L., D. Lehman, M. C. Singhal, J. Cantera, J. Singleton, P. Labarre, A. Toyama, O. Piepenburg, M. Parker, R. Wood, J. Overbaugh and D. S. Boyle (2014). "Non-Instrumented Incubation of a Recombinase Polymerase Amplification Assay for the Rapid and Sensitive Detection of Proviral HIV-1 DNA." PLoS ONE 9(9): e108189 DOI: journal.pone.0108189.

Linnes, J. C., A. Fan, N. M. Rodriguez, B. Lemieux, H. Kong and C. M. Klapperich (2014). "Paper-based molecular diagnostic for Chlamydia trachomatis." RSC advances 4(80): 42245-42251 DOI: c4ra07911f.

Loo, J. F., P. M. Lau, H. P. Ho and S. K. Kong (2013a). "An aptamer-based bio-barcode assay with isothermal recombinase polymerase amplification for cytochrome-*c* detection and anti-cancer drug screening." Talanta 115: 159-165 DOI: j.talanta.2013.04.051.

Loo, J. F. C., P. M. Lau and S. K. Kong (2013b). "An aptamer-based bio-barcode assay with isothermal recombinase polymerase amplification for cytochrome-*c* detection and anti-cancer drug screening." Talanta 115: 159-165.

Lutz, S., P. Weber, M. Focke, B. Faltin, J. Hoffmann, C. Muller, D. Mark, G. Roth, P. Munday, N. Armes, O. Piepenburg, R. Zengerle and F. von Stetten (2010). "Microfluidic lab-on-a-foil for nucleic acid analysis based on isothermal recombinase polymerase amplification (RPA)." Lab Chip 10(7): 887-893 DOI: b921140c.

Mabey, D., R. W. Peeling, A. Ustianowski and M. D. Perkins (2004). "Tropical infectious diseases: Diagnostics for the developing world." Nat Rev Micro 2(3): 231-240.

Mahony, J., S. Chong, D. Bulir, A. Ruyter, K. Mwawasi and D. Waltho (2013). "Multiplex loop-mediated isothermal amplification (M-LAMP) assay for the detection of influenza A/H1, A/H3 and influenza B can provide a specimen-to-result diagnosis in 40 min with single genome copy sensitivity." J Clin Virol 58(1): 127-131 DOI: j.jcv.2013.06.006.

McHugh, T. D., C. F. Pope, C. L. Ling, S. Patel, O. J. Billington, R. D. Gosling, M. C. Lipman and S. H. Gillespie (2004). "Prospective evaluation of BDProbeTec strand displacement amplification (SDA) system for diagnosis of tuberculosis in non-respiratory and respiratory samples." J Med Microbiol 53(Pt 12): 1215-1219 DOI: jmm.0.45780-0.

Mekuria, T. A., S. Zhang and K. C. Eastwell (2014). "Rapid and sensitive detection of Little cherry virus 2 using isothermal reverse transcription-recombinase polymerase amplification." J Virol Methods 205C: 24-30 DOI: j.jviromet.2014.04.015.

Meridian Bioscience. "Products: illumigene® Molecular Diagnostic System " Retrieved 24 July, 2013, from www.meridianbioscience.com/diagnostic-products/group-b-streptococcus/illumigene-molecular-diagnostic-system/illumigene-gbs.aspx.

Ming, K., J. Kim, M. J. Biondi, A. Syed, K. Chen, A. Lam, M. Ostrowski, A. Rebbapragada, J. J. Feld and W. C. Chan (2015). "Integrated Quantum Dot Barcode Smartphone Optical Device for Wireless Multiplexed Diagnosis of Infected Patients." ACS Nano DOI: nn5072792.

Moore, C. (2013). "Point-of-care tests for infection control: should rapid testing be in the laboratory or at the front line?" Journal of Hospital Infection 85(1): 1-7 DOI: j.jhin.2013.06.005.

Morel, P., A. Stasiak, S. D. Ehrlich and E. Cassuto (1994). "Effect of length and location of heterologous sequences on RecA-mediated strand exchange." J Biol Chem 269(31): 19830-19835.

Mori, Y. and T. Notomi (2009). "Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases." J Infect Chemother 15(2): 62-69.

Murinda, S. E., A. M. Ibekwe, S. Zulkaffly, A. Cruz, S. Park, N. Razak, F. M. Paudzai, L. Ab Samad, K. Baquir, K. Muthaiyah, B. Santiago, A. Rusli and S. Balkcom (2014). "Real-time isothermal detection of Shiga toxin-producing Escherichia coli using recombinase polymerase amplification." Foodborne Pathog Dis 11(7): 529-536.

Newton, C. R., A. Graham, L. E. Heptinstall, S. J. Powell, C. Summers, N. Kalsheker, J. C. Smith and A. F. Markham (1989). "Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS)." Nucleic Acids Res 17(7): 2503-2516.

Niemz, A., T. M. Ferguson and D. S. Boyle (2011). "Point-of-care nucleic acid testing for infectious diseases." Trends Biotechnol 29(5): 240-250 DOI: j.tibtech.2011.01.007.

Notomi, T., H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino and T. Hase (2000). "Loop-mediated isothermal amplification of DNA." Nucleic Acids Res 28(12): E63.

Oh, W. (2013). "Early Onset Neonatal Group B Streptococcal Sepsis." Am J Perinatol DOI: 10.1055/s-0032-1332804.

Oyola, S. O., T. D. Otto, Y. Gu, G. Maslen, M. Manske, S. Campino, D. J. Turner, B. Macinnis, D. P. Kwiatkowski, H. P. Swerdlow and M. A. Quail (2012). "Optimizing Illumina next-generation sequencing library preparation for extremely AT-biased genomes." BMC Genomics 13: 1 DOI: 10.1186/1471-2164-13-1.

Park, J. S., D. H. Cho, J. H. Yang, M. Y. Kim, S. M. Shin, E. C. Kim, S. S. Park and M. W. Seong (2013). "Usefulness of a Rapid Real-time PCR Assay in Prenatal Screening for Group B Streptococcus Colonization." Ann Lab Med 33(1): 39-44 DOI: alm.2013.33.1.39.

Patil, K. N., P. Singh and K. Muniyappa (2011). "DNA Binding, Coprotease, and Strand Exchange Activities of Mycobacterial RecA Proteins: Implications for Functional Diversity among RecA Nucleoprotein Filaments." Biochemistry 50(2): 300-311 DOI: Bi1018013.

Petti, C. A. (2007). "Detection and identification of microorganisms by gene amplification and sequencing." Clinical Infectious Diseases 44(8): 1108-1114.

Picard, F. J. and M. G. Bergeron (2002). "Rapid molecular theranostics in infectious diseases." Drug Discov Today 7(21): 1092-1101.

Picard, F. J., M. Gagnon, M. R. Bernier, N. J. Parham, M. Bastien, M. Boissinot, R. Peytavi and M. G. Bergeron (2009). "Internal control for nucleic acid testing based on the use of purified *Bacillus atrophaeus* subsp. *globigii* spores." J Clin Microbiol 47(3): 751-757 DOI: JCM.01746-08.

Picard, F. J. and C. Menard (2009). "Universal method and composition for the rapid lysis of cells for the release of nucleic acids and their detection." http://www.google.ca/patents/US7494771.

Piepenburg, O. and N. A. Armes (2011). "DNA Glycosylase/Lyase and AP Endonuclease substrates." http://www.google.com/patents/US20110053153.

Piepenburg, O., C. H. Williams and N. A. Armes (2014). "Methods for multiplexing Recombinase Polymerase Amplification." http://www.google.com/patents/US20140295436.

Piepenburg, O., C. H. Williams, D. L. Stemple and N. A. Armes (2006). "DNA detection using recombination proteins." PLoS Biol 4(7): 1115-1121 DOI: journal.pbio.0040204.

R Project. "The R Project for Statistical Computing." Retrieved 24 July, 2013, from http://www.r-project.org/.

Ravan, H. and R. Yazdanparast (2012). "Development and evaluation of a loop-mediated isothermal amplification method in conjunction with an enzyme-linked immunosorbent assay for specific detection of Salmonella serogroup D." Anal Chim Acta 733: 64-70 DOI: j.aca.2012.04.034.

Rohrman, B. A. and R. Richards-Kortum (2015). "Inhibition of Recombinase Polymerase Amplification by Background DNA: A Lateral Flow-Based Method for Enriching Target DNA." Anal Chem DOI: ac504365v.

Rohrman, B. A. and R. R. Richards-Kortum (2012). "A paper and plastic device for performing recombinase polymerase amplification of HIV DNA." Lab on a Chip 12(17): 3082-3088 DOI: c2lc40423k.

Roy, E., G. Stewart, M. Mounier, L. Malic, R. Peytavi, L. Clime, M. Madou, M. Bossinot, M. G. Bergeron and T. Veres (2014). "From cellular lysis to microarray detection, an integrated thermoplastic elastomer (TPE) point of care Lab on a Disc." Lab on a Chip 15(2): 406-416 DOI: 10.1039/c4lc00947a.

Sakai, K., P. Trabasso, M. L. Moretti, Y. Mikami, K. Kamei and T. Gonoi (2014). "Identification of fungal pathogens by visible microarray system in combination with isothermal gene amplification." Mycopathologia 178(1-2): 11-26.

Sakallah, S. A. (2000). "Molecular diagnostics of infectious diseases: state of the technology." Biotechnol Annu Rev 6: 141-161.

Santiago-Felipe, S., L. A. Tortajada-Genaro, S. Morais, R. Puchades and A. Maquieira (2015). "Isothermal DNA amplification strategies for duplex microorganism detection." Food Chem 174: 509-515 DOI: j.foodchem.2014.11.080.

Santiago-Felipe, S., L. A. Tortajada-Genaro, S. Morais, R. Puchades and Á. Maquieira (2014a). "One-pot isothermal DNA amplification – Hybridisation and detection by a disc-based method." Sensors and Actuators B: Chemical 204(0): 273-281 DOI: http://dx.doi.org/10.1016/j.snb.2014.07.073.

Santiago-Felipe, S., L. A. Tortajada-Genaro, R. Puchades and A. Maquieira (2014b). "Recombinase polymerase and enzyme-linked immunosorbent assay as a DNA amplification-detection strategy for food analysis." Anal Chim Acta 811: 81-87 DOI: j.aca.2013.12.017.

Savir, Y. and T. Tlusty (2010). "RecA-Mediated Homology Search as a Nearly Optimal Signal Detection System." Mol Cell 40(3): 388-396 DOI: http://dx.doi.org/10.1016/j.molcel.2010.10.020.

Schrag, S., R. Gorwitz, K. Fultz-Butts and A. Schuchat (2002). "Prevention of perinatal group B streptococcal disease. Revised guidelines from CDC." MMWR Recomm Rep 51(RR-11): 1-22.

Schuurman, T., R. de Boer, R. Patty, M. Kooistra-Smid and A. van Zwet (2007). "Comparative evaluation of in-house manual, and commercial semi-automated and automated DNA extraction platforms in the sample preparation of human stool specimens for a *Salmonella enterica* 5'-nuclease assay." J Microbiol Methods 71(3): 238-245 DOI: j.mimet.2007.09.003.

Schwartz, J., B. Robinson-Dunn, J. Makin and B. L. Boyanton, Jr. (2012). "Evaluation of the BD MAX GBS assay to detect Streptococcus group B in LIM broth-enriched antepartum vaginal-rectal specimens." Diagn Microbiol Infect Dis 73(1): 97-98 DOI: j.diagmicrobio.2012.01.016.

Sharma, N., S. Hoshika, D. Hutter, K. M. Bradley and S. A. Benner (2014). "Recombinase-Based Isothermal Amplification of Nucleic Acids with Self-Avoiding Molecular Recognition Systems (SAMRS)." Chembiochem DOI: cbic.201402250.

Shen, F., E. K. Davydova, W. Du, J. E. Kreutz, O. Piepenburg and R. F. Ismagilov (2011a). "Digital isothermal quantification of nucleic acids via simultaneous chemical initiation of recombinase polymerase amplification reactions on SlipChip." Analytical Chemistry 83(9): 3533-3540 DOI: ac200247e.

Shen, F., E. K. Davydova, W. B. Du, J. E. Kreutz, O. Piepenburg and R. F. Ismagilov (2011b). "Digital Isothermal Quantification of Nucleic Acids via Simultaneous Chemical Initiation of Recombinase Polymerase Amplification Reactions on SlipChip." Analytical Chemistry 83(9): 3533-3540 DOI: Doi 10.1021/Ac200247e.

Shin, Y., A. P. Perera, K. W. Kim and M. K. Park (2013). "Real-time, label-free isothermal solid-phase amplification/detection (ISAD) device for rapid detection of genetic alteration in cancers." Lab Chip 13(11): 2106-2114 DOI: c3lc50129a.

Shin, Y., A. P. Perera, W. Y. Tang, D. L. Fu, Q. Liu, J. K. Sheng, Z. Gu, T. Y. Lee, T. Barkham and M. Kyoung Park (2015). "A rapid amplification/detection assay for analysis of Mycobacterium tuberculosis using an isothermal and silicon bio-photonic sensor complex." Biosens Bioelectron 68C: 390-396 DOI: j.bios.2015.01.030.

Siegrist, J., R. A. Gorkin, R. Peytavi, M. Madou, H. Kido, M. Amasia, E. Roy and T. Veres (2011). "Centrifugal microfluidic system for nucleic acid sample preparation, amplification, and detection " Accessed 20110111987.

Sturenburg, E. and R. Junker (2009). "Point-of-care testing in microbiology: the advantages and disadvantages of immunochromatographic test strips." Dtsch Arztebl Int 106(4): 48-54 DOI: arztebl.2009.0048.

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar (2013). "MEGA6: Molecular Evolutionary Genetics Analysis version 6.0." Mol Biol Evol 30(12): 2725-2729 DOI: molbev/mst197.

Teoh, B. T., S. S. Sam, K. K. Tan, M. B. Danlami, M. H. Shu, J. Johari, P. S. Hooi, D. Brooks, O. Piepenburg, O. Nentwich, A. Wilder-Smith, L. Franco, A. Tenorio and S. AbuBakar (2015). "Early detection of the dengue virus using reverse transcription-recombinase polymerase amplification." J Clin Microbiol DOI: JCM.02648-14.

Tsaloglou, M. N., R. J. Watson, C. M. Rushworth, Y. Zhao, X. Niu, J. M. Sutton and H. Morgan (2015). "Real-time microfluidic recombinase polymerase amplification for the toxin B gene of Clostridium difficile on a SlipChip platform." Analyst 140(1): 258-264 DOI: c4an01683a.

TwistDx, L. "Instruction Manuals." Retrieved 05-05, 2015, from http://www.twistdx.co.uk/resources/instruction_manuals.

TwistDx, L. "TwistAmp exo RT." Retrieved 25-06, 2015, from http://www.twistdx.co.uk/products/general_rd_kits/twistamp_exo.

TwistDx, L. (2013). "Products: TwistAmp exo, real-time fluorescent DNA detection." Retrieved 2013-07-24, 2013, from http://www.twistdx.co.uk/products/general_rd_kits/twistamp_exo/.

TwistDx, L. (2015a). "Resources/News." Retrieved 2015-03-17, 2015, from http://www.twistdx.co.uk/resources/news/.

TwistDx, L. (2015b). "TwistAmp Manuals." Retrieved 2015-05-05, 2015, from http://www.twistdx.co.uk/resources/instruction_manuals/twistamp_product_documentation/.

U.S. Food and Drug Administration. (2015). "Nucleic Acid Based Tests." List of Microbial Tests Retrieved 08-04-2015, 2015, from http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm330711.htm.

Urdea, M., L. A. Penny, S. S. Olmsted, M. Y. Giovanni, P. Kaspar, A. Shepherd, P. Wilson, C. A. Dahl, S. Buchsbaum, G. Moeller and D. C. Hay Burgess (2006). "Requirements for high impact diagnostics in the developing world." Nature 444 Suppl 1: 73-79 DOI: nature05448.

Van Ness, J., L. K. Van Ness and D. J. Galas (2003). "Isothermal reactions for the amplification of oligonucleotides." Proc Natl Acad Sci U S A 100(8): 4504-4509 DOI: pnas.0730811100.

Verani, J. R., L. McGee and S. J. Schrag (2010). "Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010." MMWR Recomm Rep 59(RR-10): 1-36.

Verheyen, J., R. Kaiser, M. Bozic, M. Timmen-Wego, B. K. Maier and H. H. Kessler (2012). "Extraction of viral nucleic acids: comparison of five automated nucleic acid extraction platforms." J Clin Virol 54(3): 255-259 DOI: j.jcv.2012.03.008.

Vincent, M., Y. Xu and H. Kong (2004). "Helicase-dependent isothermal DNA amplification." EMBO Rep 5(8): 795-800.

Wee, E. J., H. Y. Lau, J. R. Botella and M. Trau (2015). "Re-purposing bridging flocculation for on-site, rapid, qualitative DNA detection in resource-poor settings." Chem Commun (Camb) DOI: c4cc10068a.

Xia, X., Y. Yu, L. Hu, M. Weidmann, Y. Pan, S. Yan and Y. Wang (2015). "Rapid detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) by real-time, isothermal recombinase polymerase amplification assay." Arch Virol DOI: 10.1007/s00705-015-2357-7.

Xia, X., Y. Yu, M. Weidmann, Y. Pan, S. Yan and Y. Wang (2014). "Rapid Detection of Shrimp White Spot Syndrome Virus by Real Time, Isothermal Recombinase Polymerase Amplification Assay." PLoS ONE 9(8): e104667 DOI: journal.pone.0104667.

Xu, C., L. Li, W. Jin and Y. Wan (2014a). "Event-specific Real-time RPA Detection of Transgenic Rice Kefeng 6." GMO Biosafety Research 5(1): 1-5.

Xu, C., L. Li, W. Jin and Y. Wan (2014b). "Recombinase Polymerase Amplification (RPA) of CaMV-35S Promoter and nos Terminator for Rapid Detection of Genetically Modified Crops." Int J Mol Sci 15(10): 18197-18205 DOI: ijms151018197.

Yan, L., J. Zhou, Y. Zheng, A. S. Gamson, B. T. Roembke, S. Nakayama and H. O. Sintim (2014). "Isothermal amplified detection of DNA and RNA." Mol Biosyst 10(5): 970-1003 DOI: c3mb70304e.

Yang, G., D. E. Erdman, M. Kodani, J. Kools, M. D. Bowen and B. S. Fields (2011). "Comparison of commercial systems for extraction of nucleic acids from DNA/RNA respiratory pathogens." J Virol Methods 171(1): 195-199 DOI: j.jviromet.2010.10.024.

Zhang, S., M. Ravelonandro, P. Russell, N. McOwen, P. Briard, S. Bohannon and A. Vrient (2014). "Rapid diagnostic detection of plum pox virus in Prunus plants by isothermal AmplifyRP using reverse transcription-recombinase polymerase amplification." J Virol Methods 207C: 114-120 DOI: j.jviromet.2014.06.026.

Zhang, Z., D. Yoon, J. R. LaPorte and J. Chen (2001). "Appropriate initiation of the strand exchange reaction promoted by RecA protein requires ATP hydrolysis." J Mol Biol 309(1): 29-43 DOI: jmbi.2001.4753.

Appendix 1

Chapter I supplemental table & figures

Table1.4. MuLV RT-RPA LOD

	Presence of Nasopharyngeal pool specimens		Absence of Nasopharyngeal pool specimens	
RNA transcript copy number	Positive replicate	Mean time threshold (min)	Positive replicate	Mean time threshold (min)
100	8/8	9.3	8/8	6.69
50	8/8	11.6	8/8	6.98
25	8/8	13.0	8/8	7.68
10	6/8	13.8	7/8	9.29
5	4/8	17.3	7/8	10.95
LOD (95%CI)*	13.5 ± 6.6		8.77 ± 2.24	

* LOD calculation was done as published previously using Lotlod version 1.2.2 (Daher, Stewart *et al.* 2014).

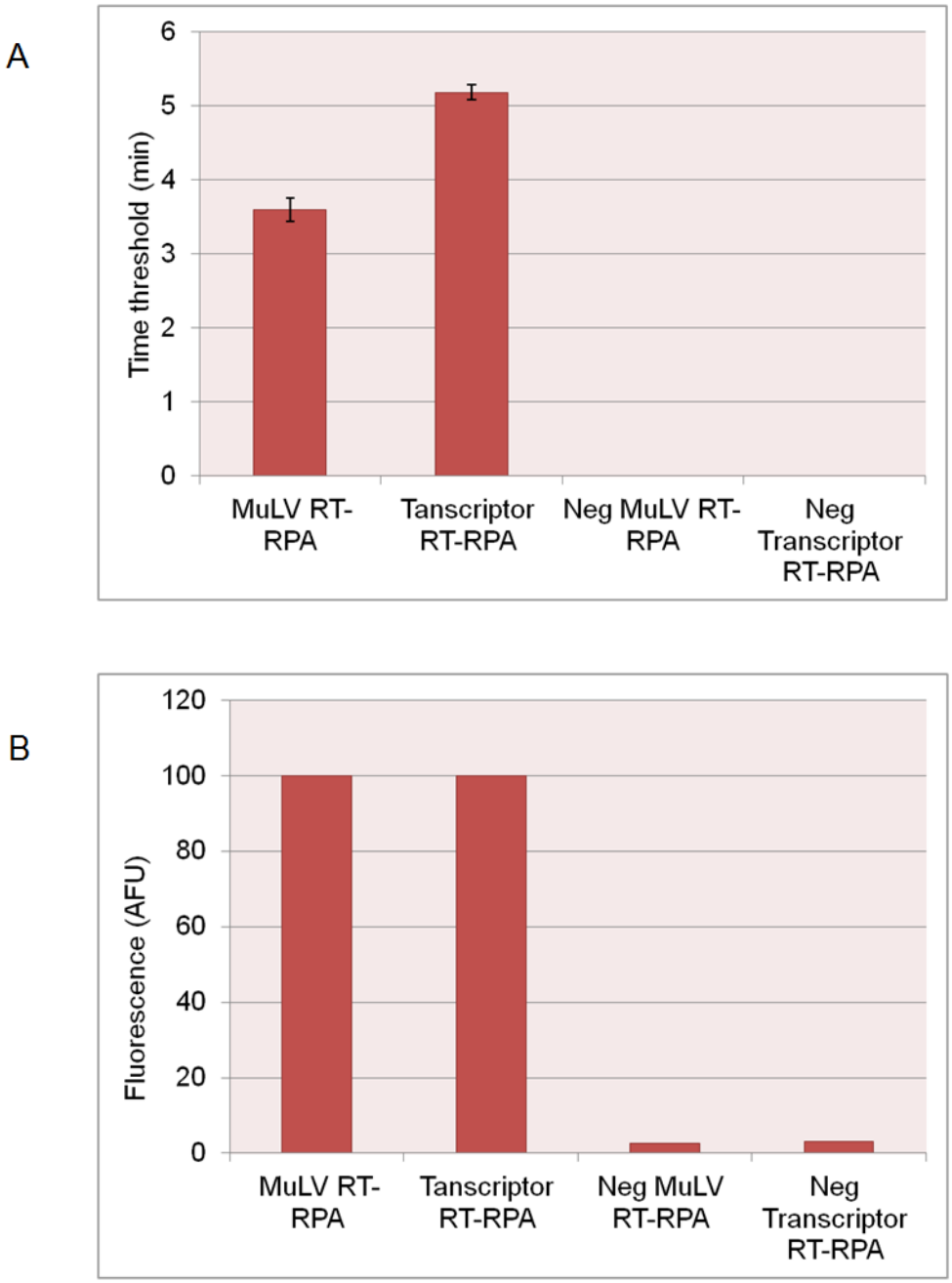

Figure 1.7. Evaluation of MuLV RT-RPA to Transcriptor RT-RPA

(A) Mean time threshold for amplification signals obtained by Rotor-Gene normalized fluorescence curves of Influenza A RNA transcript (10^4 copies) amplified with MuLV RT from the TwistAmp™ exo RT kit versus Transcriptor RT-RPA (Transcriptor RT added to TwistAmp™ exo kit). Standard error of the mean shows the variability of time threshold for sample. (B) End-point fluorescence intensity for amplification signal of RNA transcript amplified with MuLV RT-RPA versus Transcriptor RT-RPA.

Appendix 2

Chapter V supplemental figure

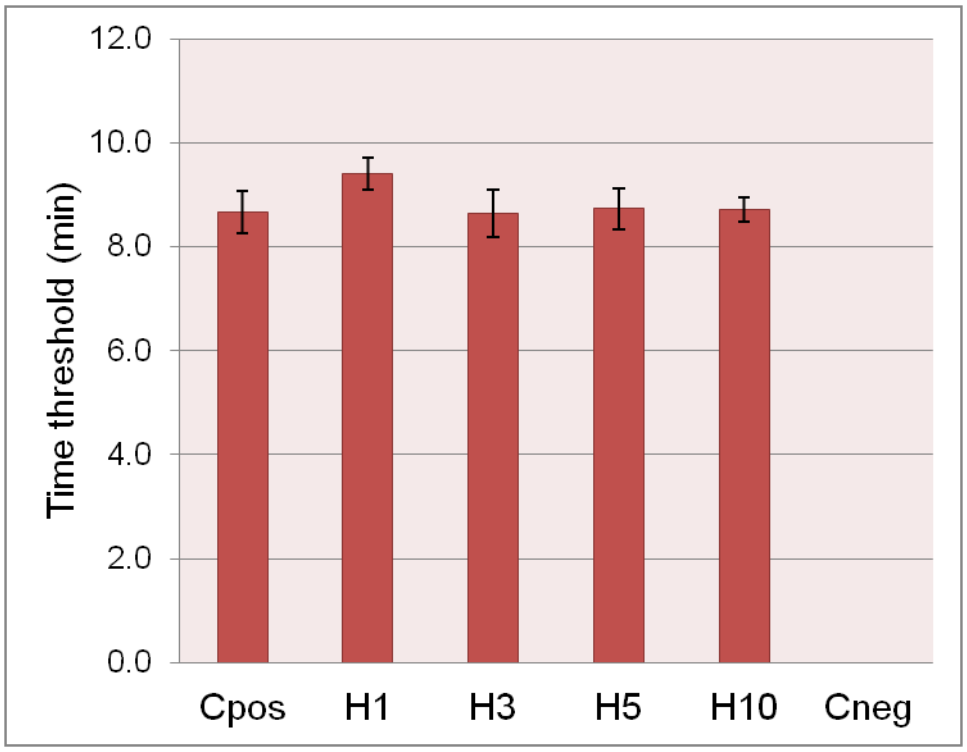

Figure 5.7. Stability of RPA lyophilized reaction pellet

Mean time threshold for amplification signals obtained by Rotor-Gene normalized fluorescence curves of 10^3 copies of GBS genomic DNA amplified with fresh TwistAmp™ exo RPA lyophilized pellet versus heat-subjected RPA pellets at different duration time. Standard error of the mean shows the low variability of time threshold between fresh and heat-subjected samples. Cpos = fresh RPA pellet; H1= RPA pellet heated for 1 min at 95°C; H3 = RPA pellet heated for 3 min at 95°C; H5 = RPA pellet heated for 5 min at 95°C; H10 = RPA pellet heated for 10 min at 95°C; Cneg = non template control.

Appendix 3

Chapter VI supplemental figure

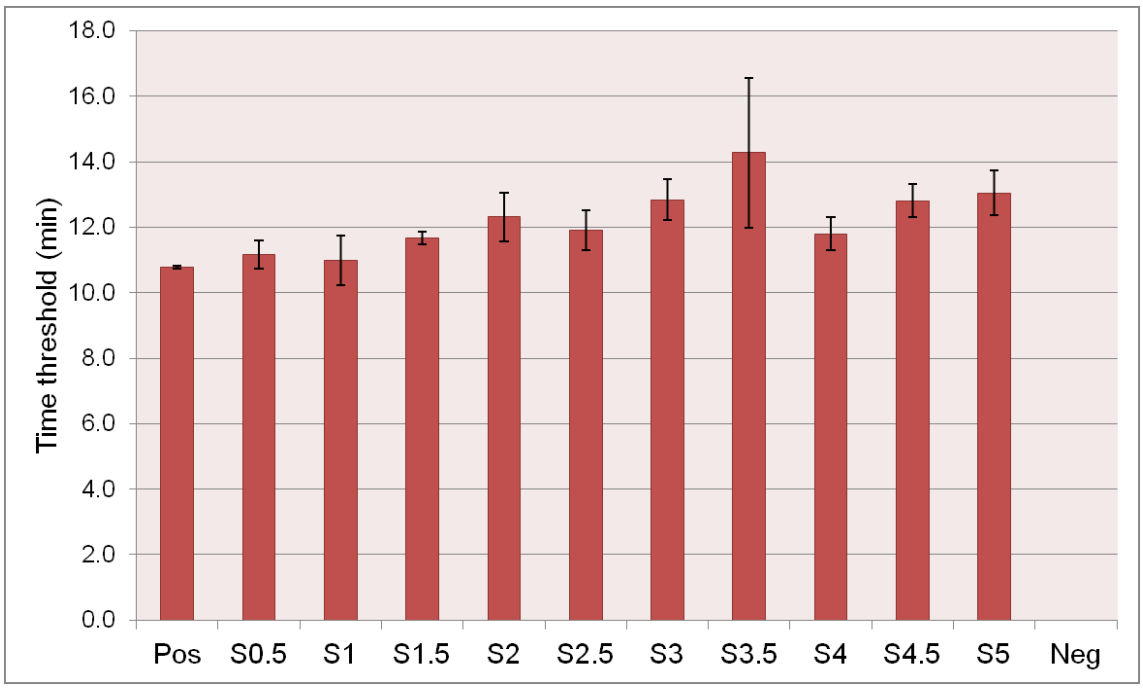

Figure 6.1. RPA performance with stool samples

Mean time threshold for amplification signals obtained by Rotor-Gene normalized fluorescence curves of 10^3 copies of GBS genomic DNA amplified with or without the addition of different volumes of untreated stool samples (*i.e.* 0.5 to 5 µl added to the RPA reaction) that were GBS negative: S0.5 = 0.5 µl spiked stool; S1= 1 µl spiked stool, and so on. Standard error of the mean shows the variability of time threshold of different samples. Pos = positive control, 10^3 copies GBS genomic DNA; Neg = non template control.

Appendix 4

Posters' Abstracts

Poster presented at

- ***Journée scientifique du centre de recherche du CHU de Québec***, Québec City, Canada, October 20, 2014.

- ***15e Journée annuelle de la recherche de la Faculté de médecine***, Université Laval, Québec City, Canada, May 30, 2013.

Résumé

Étude comparative de la "recombinase polymerase amplification" à la PCR dans la détection rapide des échantillons cliniques de Streptocoque de Groupe B

Rana K. Daher[1, 2], Gale Stewart[2], Maurice Boissinot[1, 2] et Michel G. Bergeron[2]

[1]Université Laval, Québec, Qc, Canada, [2]Centre de recherche du CHU de Québec, Centre de recherche en infectiologie du CHUL, Québec, Qc, Canada

Objectif: Nous avons démontré dans des études antérieures la performance de la PCR en temps réel (rt-PCR) comme outil de diagnostic pour la détection rapide en moins d'une heure du Streptocoque de Groupe B (GBS). Dans cette étude, nous avons comparé la performance d'une technique d'amplification isotherme nommée "recombinase polymerase amplification" (RPA) à la rt-PCR pour l'identification rapide du GBS.

Méthodes: Des lysats de 50 échantillons cliniques congelés prélevés sur des femmes enceintes au moment de l'accouchement ont été amplifiés par rt-PCR ainsi que par RPA. La sensibilité et spécificité clinique de la RPA sont calculées par rapport à la rt-PCR comme méthode de référence. La limite de détection (LOD) de la RPA est déterminée en faisant des dilutions sériées de l'ADN génomique de GBS et estimée par le logiciel Lotlod (version 1.2.2). La spécificité analytique de la RPA est déterminée en testant une variété de souches bactériennes/fungiques naturellement présentes dans la flore vaginale/anale.

Résultats: Parmi les 50 lysats d'échantillons cliniques congelés et analysés, 25 sont GBS positifs et 25 autres sont GBS négatifs par la méthode rt-PCR. Un seul échantillon des 25 positifs en rt-PCR est manqué par la RPA ce qui donne une sensibilité clinique de 96%. Les 25 échantillons négatifs en rt-PCR sont aussi négatifs en RPA (spécificité clinique de 100%). Le calcul fait par Lotlod montre une LOD de 98±20 copies de génome dans 95% des cas. La spécificité analytique de la RPA est à 100%, aucune des souches bactériennes/fungiques à l'étude n'a été détectée. Le temps pour obtenir un résultat en RPA est moins de 20 min versus 45 min habituellement pour la rt-PCR.

Conclusions: La RPA est comparable en performance à la rt-PCR. Celle-ci surpasse la rt-PCR en vitesse pour obtenir le résultat ce qui en fait un outil de diagnostic clinique potentiel pour la détection ultra-sensible et rapide des microorganismes tels que GBS.

❖ **Poster presented at**

- 113th American Society for Microbiology General Meeting 2013, Mai 18-21, 2013, Denver, Colorado.

Abstract

First Comparison of Recombinase Polymerase Amplification Assay with Real-Time PCR for the Rapid Detection of Group B Streptococci from Clinical Samples

Rana K. Daher[1, 2], Gale Stewart[2], Maurice Boissinot[1, 2] and Michel G. Bergeron[2]

[1]Université Laval, Québec, Qc, Canada, [2]Centre de recherche du CHU de Québec, Centre de recherche en infectiologie du CHUL, Québec, Qc, Canada

Background: Group B streptococcal (GBS) infections are the leading cause of sepsis and meningitis in newborns. The need for a rapid and a reliable method for the detection of GBS at the time of delivery is important for the early treatment of neonates. Isothermal amplification techniques such as recombinase polymerase amplification (RPA) showed some advantages relative to PCR in terms of the speed of reaction and simplicity.

Materials: We studied the performance of RPA for the screening of 50 frozen vaginal/anal lysates collected from pregnant women. The specimens were also tested for GBS by real-time PCR (rt-PCR) using BD GeneOhm™ Strep B assay. In addition, both analytical sensitivity and specificity of the RPA assay were compared to PCR.

Results: Among the 50 specimens, 25 were negative for GBS and 25 others were positive as determined by the rt-PCR assay. The RPA assay detected 24 positive samples out of 25 (96%) and 25 negative out of 25 (100%). The analytical sensitivity or limit of detection (LOD) of the RPA assay was 98 genome copies compared to 25 genome copies claimed for the BD GeneOhm™ Strep B assay. The analytical specificity for RPA was 100% for a panel of 10 bacterial and/or fungal strains naturally found in the vaginal/anal flora. Time-to-result was less than 20 minutes for the RPA assay compared to 45 min for the rt-PCR assay.

Conclusion: The screening of GBS in pregnant women can be performed rapidly by the isothermal amplification technique, RPA assay. However, in our hands this isothermal technique lacked sensitivity compared to PCR. Therefore, RPA needs to be improved for future clinical application purposes.