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Rolling Circle Amplification (RCA): an approach for quick detection and identification of fungal species

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Abstract:

Conventional methods for fungal identification in the clinical laboratory rely on morphological and physiological tests. These tests often need several days or weeks to complete and are frequently unspecific. Molecular identification mostly implies sequencing, which is relatively expensive and time-consuming, as well as impractical for large numbers of isolates. The Rolling Circle Amplification approach, known as RCA, is a quick, critical and economic method for fungal species' identification. Despite its high speed, this method is highly sensitive, and it has been widely used for the detection of pathogenic fungi. The specific probes are designed based on the differences in the nucleotide regions of the target gene for the target species. The amplification product can be visualized by agarose gel electrophoresis, but can also be visualized in gel-free systems using fluorescence staining of the amplified product by SYBR Green in combination with a UV transilluminator. Thus, the simplicity, sensitivity, robustness and low costs make RCA an attractive technique for the reliable identification of sibling species and other closely related fungi.

Keywords: Padlock probes, Rolling Circle Amplification (RCA), Single nucleotide polymorphisms.

Introduction

It seems fair to say that many systematists agree that fungi species are real, important and sometimes extremely difficult to identify. There is also a growing sense that the virtually limitless empirical data available from emerging genomic databases may help solve the problem of delimiting difficult, recently derived species (Shaffer and Thomson, 2007). By creating a gene and protein bank, the researchers have found some methods that utilize the help of the new science of Bioinformatics to provide more accurate, specific and faster techniques than the conventional molecular techniques, which are very expensive and require skilled professionals in Diagnostic Sciences (Brown, 2010; Zhou et al., 2008). Today, some of the methods which have been receiving more attention from researchers are the Isothermal methods, in which DNA replication is made possible without the need to apply a thermal cycler. RCA is one such method, which has received most attention because of its ultra-high specificity. In RCA, a Padlock probe is used to identify the SNPs (Single-nucleotide polymorphisms) of the genome and since it creates a closed form in binding to a target DNA, it almost completely removes the risk of non-specific sequences replication (Gusev et al., 2001; Moradi et al., 2008; Schweitzer and Kingsmore, 2001; Yoshida et al., 2005).

RCA: Rolling Circle Amplification (RCA) is a sensitive, specific and reproducible isothermal

DNA amplification technique used for rapid molecular identification of microorganisms (Najafzadeh et al., 2013) which has gained great attention over the past decade (Wang et al., 2014b; Kuhn et al.; 2002). RCA technology, which has an intrinsically wide dynamic range, involves a robust and simple procedure that can provide a universal platform for the localization of a wide variety of molecules as a function of either antigenicity or nucleic acid sequence (Gusev et al., 2001). RCA is particularly useful to discriminate closely related species or genotypes within species, which may differ by only single nucleotide differences (Sun et al., 2011). RCA is based on the rolling replication of short single-stranded DNA (ssDNA) circular molecules (Lizardi et al., 1998; Fire and Xu, 1995; Najafzadeh et al., 2011) by certain DNA polymerases at a constant temperature, which only requires a simple platform, such as heating blocks or a water bath (Tsui et al., 2011a). This process discovered in the mid-1990s (Nilsson, 2006; Kobori and Takahashi, 2014; Demidov, 2005). The development of RCA probes to distinguish single species or groups of species relies on the presence of sufficient sequence data and useful species-specific polymorphisms in the genes of correctly identified species (Davari et al., 2012). The ligation allows efficient distinction among sequence variants and can efficiently be utilized for detection of single nucleotide polymorphisms, as DNA ligase will ligate the two ends of the probes only in cases of a perfect match with the target (Jehan and Lakhanpaul, 2006). Furthermore, the ligation reaction is sensitive to mismatches between the probes and the target (Wang et al., 2005; Kaocharoen et al., 2008a; Zhou et al., 2008; Tsui et al., 2010a; Tsui et al., 2013). RCA uses a strand-displacing DNA polymerase to continuously amplify a circular DNA template at a constant low temperature, producing a long DNA molecule with tandem repeats of the circular template (Asiello and Baeumner, 2011). Deoxynucleotides (dNTPs) are added to extend a primer bound to a single-stranded circular template, by DNA polymerases possessing strand displacement activity. This gives rise to a long single-stranded fragment of DNA comprising concatemers of the original circular template (Pang et al., 2007). RCA involves an initial forward primer that binds to the padlock probe and initializes RCA, and a second primer that targets the repeated ssDNA sequence of the primary RCA product, finally generating large numbers of copies of the DNA fragments. This is called hyperbranching RCA (H-RCA) (Tsui et al., 2011a; Pang et al., 2007; Lizardi et al., 1998; Tsui et al., 2013). Accordingly, the geometric RCA is more potent, as compared with its linear alternative, yielding 109 or more copies of a circular sequence in about an hour (Demidov, 2005). Non-circularized probes are removed by exonuclease treatment, while the circularized ones may be amplified by using universal primers (Szemes et al., 2005). By increasing the hybridization temperature and shortening the 3' arm (below the reaction temperature), the discrimination of SNP can be further improved (Faruqi et al., 2001; Tsui et al., 2011a) and precise amounts of RCA product can be generated that are dependent upon the quantity of dNTPs incorporated into the reaction mixture (Pang et al., 2007). Due to the drastic signal amplification power, RCA has been widely employed in various sensing schemes for the analyses of proteins and nucleic acids (Li et al., 2008; Wang et al., 2014a). The RCA reactions have been run on the single-stranded DNA and RNA targets, and also, with the aid of PNA openers, these reactions can be performed with dsDNA (Demidov, 2005). The duration of the RCA assay was two hours (Najafzadeh et al., 2013), however, a positive signal was usually evident within 15 minutes after onset of RCA reaction when performed by real time PCR (Sun et al., 2011). The entire process, including DNA extraction, PCR amplification, ligation of padlock probes, exonucleolysis, RCA itself and gel electrophoresis could be finished within one working day (Sun et al., 2011).

Materials and Methods

DNA extraction and amplification: DNA extraction protocols vary with the samples used.

The ITS region is widely used as a target sequence for the identification of pathogenic fungi. However, whereas many fungi show insufficient diversity in ITS, hypervariable partial genes and introns can be used, such as tubulin, actin, translation elongation factor1-α. The amplicons were generated with commercial primers following the manufacturer's instructions.

Padlock probe design: In order to design a padlock probe, at first we selected a gene with enough resolution as the target for the padlock probe design (Tsui et al., 2013). To ensure the efficiency of the padlock probe binding, the padlock probes were designed with minimum secondary structure and with the Tm of the 5' end probe binding arm close to or above the ligation temperature (63 °C) (Feng et al., 2013; Najafzadeh et al., 2013; Najafzadeh et al., 2011). To increase its discriminative specificity, the 3' end binding arm was designed with a Tm 10°C-15°C below ligation temperature (Najafzadeh et al., 2013; Najafzadeh et al., 2011) and specificity can be increased by selecting polymorphisms in the 3' end binding arm (Najafzadeh et al., 2013). The genetic linker region was also carefully designed to minimize any similarity to potentially cross-reacting sequences after BLAST search. The specificity of the probes was confirmed by BLAST analysis in GenBank (Sun et al., 2011).

A padlock probe refers to long oligonucleotides (about 100 bp) (Tsui et al., 2011a), comprising (i) a 5´-phosphorylated end, (ii) a "backbone" containing binding sites for the RCA primers (RCA primers 1 and 2, respectively; designated by bold uppercase letters) as well as the nonspecific linker regions (designated by bold lowercase letters), and (iii) a 3´ end. The 5´ and 3´ ends of the probe are complementary to the 5´ and 3´ termini of the target sequence in reverse (Zhou et al., 2008; Tong et al., 2007). Phosphate groups were added at the 5' ends of the molecules as required for enzymatic ligation (Nilsson et al., 1994). The basic structure of a padlock probe is depicted in Fig. 1.

Ligation of the padlock probe: One microliter of gene amplicon was mixed with 2 U pfu DNA ligase (Epicentre Biotechnologies, Madison, WI, USA) and 0.1 µmol lµ1 padlock probe in 20 mmol lµ1 Tris-HCl (pH 7.5), 20 mmol lµ1 Cl, 10 mmol lµ1 MgCl2, 0.1% Igepal, 0.01 mmol lµ1 rATP, and 1 mmol lµ1 DTT, with a total reaction volume of 10 µl. Padlock probe ligation was conducted with one cycle of denaturation for five minutes at 94°C, followed by five cycles of 94°C for 30 seconds and four minutes of ligation at 63°C.

Exonucleolysis: Exonucleolysis is required to remove an unligated padlock probe and template PCR product and thus reduces subsequent ligation-independent amplification events. This was performed in a 20-µl vol by addition of 10 U each of exonuclease I and III (New England Biolabs, Hitchin, UK) to the ligation mixture and incubation at 37°C for 30 minutes, followed by 94°C for three minutes to deactivate the exonuclease reaction.

Rolling Circle Amplification (RCA) reaction: Two microliters of ligation product were used as a template for RCA. The total volume was 50 µl containing 8 U Bst DNA polymerase (New England Biolabs), 400 µmol lµ1 deoxynucleoside triphosphate mix, and 10 pmol of each RCA primer in distilled water. Probe signals were amplified by incubation at 65°C for 60 minutes.

Data Analysis: The RCA amplicons can be detected using several methods, such as fluorescence (Szemes et al., 2005), radiolabeling (Banér et al., 1998), UV absorbance (Kuhn et al., 2002), and gel electrophoresis (Sun et al., 2011), by using either direct incorporation of various labels into the RCA products (Banér et al., 1998) or label-decorated amplicons (Schweitzer and Kingsmore, 2001) and colorimetrically (Ali and Li, 2009). The simplest method is gel electrophoresis on a 1% agarose gel to verify the specificity of probe-template binding. Positive reactions showed a ladder-like pattern, whereas negative reactions showed a clean background.

Applications of RCA: The RCA technology is promising for molecular diagnostic and pharmacogenomic use (Kuhn et al., 2002). To date, RCA has mainly been used for the detection of viruses (Wang et al., 2005, Schubert et al., 2007) and bacteria (Tong et al., 2007). The RCA technique has successfully been applied to

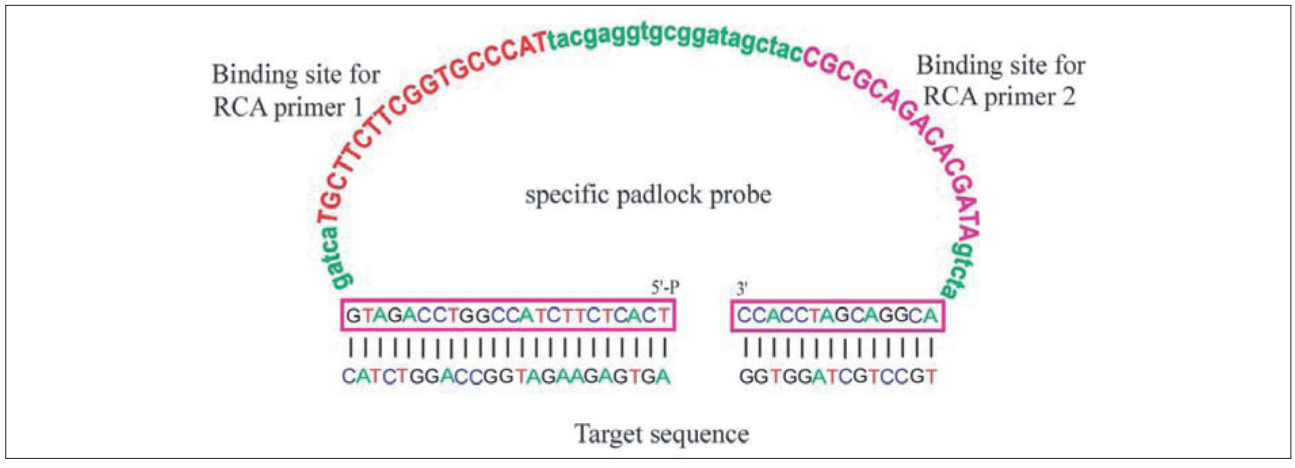

Fig. 1. Schematic representation of a padlock probe.

different fungal species like *Candida*, *Aspergillus*, and *Scedosporium* spp., *Cyphellophora* and relatives, *Fonsecaea* spp., *Exophiala* spp., *Trichophyton* spp., *Penicillium marneffei*, *Cladophialophora* carrionii, Pneumocystis jirovecii, *Fusarium* spp.and *Cryptococcus* species (Zhou et al., 2008; Najafzadeh et al., 2011; Sun et al., 2011; Kong et al., 2008; Tong et al., 2007; Feng et al., 2013; Lackner et al., 2012; Hamzehei et al., 2013; Tsui et al., 2010b; Chen and Kong, 2007; Tsui et al., 2011b; Kaocharoen et al., 2008b; Eriksson et al., 2009). RCA provides a powerful tool for a rapid and specific identification of fungi in the clinical laboratory and offers significant potential for ecological studies (Sun et al., 2011). The RCA potential to identify nucleic acid targets, antibodies and antigens in clinical samples has recently been demonstrated in several feasibility studies (Demidov, 2005). RCA-mediated multiplex profiling of cytokines on microarrays with femtomolar sensitivity offers an advantageous approach for proteomic surveys (Demidov, 2005; Li et al., 2008; Banér et al., 1998). RCA can be used for mitochondrial DNA visualization in cells immobilized on a glass substrate (Kobori and Takahashi, 2014). RCA can also be used for accurate and sensitive detection of allergens in food, which is imperative for eliminating potential health hazards triggered by food allergies (Kobori and Takahashi, 2014). Combining RCA with electrical DNA detection produces results in readout with a very high signal-to-noise ratio, an essential feature for sensitive and specific detection assays (Russell et al., 2014). RCA could enhance the use of markers of current interest, as well as permit the integration of emerging information from genomics and proteomics into cell- and tissue-based analyses (Gusev et al., 2001). This technique has been employed for the detection of single nucleotide polymorphisms (SNPs) within DNA fragments, forming the basis of diagnosis for numerous disease states (Pang et al., 2007). The method has been applied for amplified detection of viral RNA from tissue samples and for preparative in vitro synthesis of catalytic antisense RNA (Banér et al., 1998). RCA has also been combined with magnetic beads and reporter DNA probes in a sandwich assay to detect viral DNA (Asiello and Baeumner, 2011).

Advantages and limitations: (RCA has several substantial advantages over other amplification techniques, as follows): RCA is sensitive (Demidov, 2005; Feng et al., 2013; Najafzadeh et al., 2013; Pang et al., 2007; Davari et al., 2012; Tsui et al., 2010a; Kong et al., 2008; Kuhn et al., 2002), specific (Najafzadeh et al., 2013; Kong et al., 2008; Feng et al., 2013; Russell et al., 2014; Najafzadeh et al., 2011), rapid (Feng et al., 2013; Najafzadeh et al., 2013; Tsui et al., 2010a; Kong et al., 2008; Wang et al., 2014a; Najafzadeh et al., 2011), inexpensive (Najafzadeh et al., 2013; Demidov, 2005), cost effective (Feng et al., 2013; Davari et al., 2012), easily operated (Feng et al., 2013; Wang et al., 2014b), contamination resistant (Kobori and Takahshi, 2014), highly efficient (Wang et al., 2014a), flexible (Kong et al., 2008)

and more error-proof (compared to PCR) (Demidov, 2005). RCA does not require highly specialized equipment, while it is relatively easily expanded to multiple or routine identifications (Davari et al., 2012). RCA is reproducible, thus reducing the chance of false positives (Najafzadeh et al., 2013; Demidov, 2005; Tsui et al., 2010a; Kong et al., 2008; Najafzadeh et al., 2011). RCA can also be performed under isothermal conditions and does not require thermal cycling (Kobori and Takahashi, 2014; Asiello and Baeumner, 2011; Pang et al., 2007; Demidov, 2005; Li et al., 2008) and makes RCA readily adaptable to routine clinical use with fewer issues concerning quality control of the instrument (Li et al., 2008). RCA can be performed by a larger variety of DNA polymerases compared to PCR, which only relies on thermostable enzymes (Demidov, 2005). Interpretation of the results is straightforward and is based on a simple positive or negative result (Tsui et al., 2010a). The method's simplicity, large multiplex potential, immunity to false positives/ cross-contamination and easy compatibility with other detection/ imaging techniques are key advantages (Kuhn et al., 2002). Furthermore, the RCA technology provides a faster, more sensitive and economical option to the currently available PCR-based methods (Wang et al., 2005). The most distinguished feature of RCA is that it can be easily carried out on a chip for high-throughput detections (Feng et al., 2013; Wang et al., 2014b). Arguably the main advantage of RCA is that it can be performed under isothermal conditions with minimal reagents and that it avoids the generation of false-positive results, a problem that is frequently encountered in PCR-based assays (Wang et al., 2005). All these unique properties of RCA facilitate its application in different research and molecular diagnosis areas like in situ detection, microarray, immunoassay, SNP, etc. (Li et al., 2008). The shortcoming of supersensitive RCA assays is that they require certain caution to avoid possible contamination/false positives (Demidov, 2005).

Conclusion

Rapid and accurate detection and identification of fungal pathogens at the species and subspecies Level both in the clinical setting (Szemes et al., 2005) and natural environment, or on plant materials, are the keys to proper patient treatment and disease/pathogen surveillance, containment and eradication. However, many fungal pathogens exist as species-complexes or they have very low abundance in the clinical specimen and natural environment (Tsui et al., 2013). Furthermore, sensitive and selective detection of sequence-specific DNA has become increasingly important in modern life science owing to its potential applicability, ranging from genetic research of diseases to clinical diagnosis and therapy (Wang et al., 2014a). In addition, the genome information of many species has been revealed; DNA-based analyses have been crucial in many biotechnology industries in the medical- and food-related sectors (Kobori and Takahashi, 2014). Furthermore, as a result of the padlock probes being used as a means of combining pathogen-specific molecular recognition and universal amplification, increasing sensitivity and multiplexing capabilities without limiting the range of potential target organisms has been achieved (Szemes et al., 2005). Due to these robustness and simplicity characteristics, the RCA-based assays hold a distinct position in the area of molecular diagnostics among other single-temperature amplification techniques (Demidov, 2005). Therefore, it is recommended to use the RCA technique as an easy and practical method with a distinct position among isothermal techniques, for DNA diagnostics as a very practical identification method (Najafzadeh et al., 2011).

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