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A simple nucleic acid hybridization/latex agglutination assay for the rapid detection of polymerase chain reaction amplicons

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Abstract

We have developed a new method for the detection of nucleic acid hybridization, based on a simple latex agglutination test that can be evaluated by the unaided eye. Nucleic acid, e.g., a polymerase chain reaction (PCR) product, is denatured and incubated with polystyrene beads carrying covalently bound complementary oligonucleotide sequences. Hybridization of the nucleic acids leads to aggregation of the latex particles, thereby verifying the presence of target sequence. The test is performed at room temperature, and results are available within 10 min. As a proof of principle, the hybridization/latex agglutination assay was applied to the detection of purified PCR fragments either specific for *Salmonella* spp. or a synthetic sequence, and to the detection of *Salmonella enterica* in artificially contaminated chicken samples. A few nanograms of purified PCR fragments were detectable. In artificially contaminated chicken samples, 3 colony-forming units (cfu)/25 g were detected in one of three replicates, and 30 cfu/25 g were detected in both of two replicates when samples for PCR were taken directly from primary enrichment, demonstrating the practical applicability of this test system. Even multiplex detection might be achievable. This novel kind of assay could be useful for a range of applications where hybridization of nucleic acids, e.g., PCR fragments, is to be detected.

Keywords: PCR; Amplicons; Hybridization; Latex agglutination; Microspheres; Detection; Pathogenic microorganisms; *Salmonella*

1. Introduction

Since the invention of Polymerase Chain Reaction (PCR), this method was extensively used for the amplification of nucleic acids in a large array of applications (see Espy et al., 2006; Yang and Rothman, 2004 for an overview). The same is true for latex agglutination tests, which have a long standing history of many decades of application, especially in the detection of micro-organisms pathogenic to humans (Kaldor et al., 1977; Leinonen and Herva, 1977), and have become widely used by both analysts and researchers as an analysis and research tool (Ortega-Vinuesa and Bastos-Gonzalez, 2001). Both methods can be used to detect pathogenic microorganisms in foodstuffs, thereby considerably shortening the detection time compared to conventional standard microbiological and biochemical methods, which are time-consuming and laborious. Whereas PCR is targeting nucleic acid, most of the existing latex agglutination tests detect proteins by means of antibody-coated microspheres. However, microspheres with nucleic acids attached to their surface have also been used for almost 20 years to develop reagents for the fractionation and characterization of nucleic acids (Wolf et al., 1987), for the detection of bacteria (Miller et al., 1988), or for studying hybridization (Lund et al., 1988). During the recent years, a multitude of innovative methods to detect PCR products or other nucleic acid targets, employing either oligonucleotide-coated microspheres (using the non-covalent biotin/streptavidin system or covalent coupling) or oligonucleotide-functionalized metal nanospheres, was developed. These methods used flow cytometry (Chandler and Jarrell, 2004; Rao et al., 2003; Spiro et al., 2000), "bead-arrays" (Kohara, 2003), microarrays (Bao et al., 2005) or other formats (Nam et al., 2003; Rosi and Mirkin, 2005; Thaxton et al., 2006) for the ultrasensitive detection of targets, or were applied for quantitative PCR (Patel et al., 2000). However, many of these highly sophisticated methods require costly equipment, and therefore are not applicable routinely, especially not for food testing in the food industry, which is demanded to provide safe,

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yet low-priced food but is not enthusiastic to turn to new methods when they are too pricy, even if they are more rapid or more sensitive.

Although simple agglutination assays are much more economical and easier to perform in the laboratory than the aforementioned methods, there exist only a few publications presenting efforts to develop such assays to detect amplification products or other nucleic acid targets (Pu and Wu, 2005; Wu et al., 2004; Storhoff et al., 1998; Bains, 1998a,b). Since an ordinary thermocycler is nowadays available at reasonable costs and thus affordable as standard equipment for many laboratories, the combination of PCR and latex agglutination by hybridization could be an economic alternative to other, more expensive methods to detect PCR amplicons.

In this paper, we describe a "low-tech", instrument-free assay to detect PCR amplicons using a quick direct approach, i.e., latex agglutination mediated by nucleic acid hybridization within a few minutes and evaluation of the result by the unaided eye. The practical applicability of this assay was demonstrated by the detection of PCR amplicons from *Salmonella enterica*. The hybridization/latex agglutination system could be a time-saving and easily disposable alternative to gel electrophoresis, which is using ethidium bromide, a strongly carcinogenic substance, and takes 1–1.5 h to complete. To our knowledge, this is the first report of a rapid direct hybridization/latex agglutination assay that can be evaluated without the need for optical instruments.

2. Materials and methods

2.1. Bacterial strain and culture conditions

For *Salmonella*-specific PCR and for artificial contamination of chicken samples, *S. enterica* serotype Enteritidis PT4 (a kind gift of Dr. Leopold Reiner, Lebensmittelversuchsanstalt, Vienna, Austria) was used. The strain was cultivated on Luria–Bertani (LB) plates or as liquid culture in LB broth at 37 °C.

2.2. Oligonucleotides

PCR primers and hybridization probes were designed and analyzed applying the Generunner software, version 3.05 (Hastings Software, Inc., Hastings-on-Hudson, NY, USA). The primers, probes, and a synthetic target used for production of an amplification control were purchased from VBC-Biotech Services GmbH, Vienna, Austria. The nucleotide sequences of all oligonucleotides used in this study are shown in Table 1. The target for *Salmonella* (SAL) was the *invA* gene (GenBank accession numbers U43273, M90846) (Boyd et al., 1996; Galán et al., 1992). The human target was the KCNJ9 gene (GenBank accession number AF193615) (Vaughn et al., 2000). The sequences were analyzed for possible homologies to sequences of the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Double-stranded CON-PCR fragment for purification and subsequent use in sensitivity tests, and as an internal positive control, was synthesized by PCR (see below) using CON-primers and single-stranded synthetic target.

2.3. Covalent coupling of oligonucleotide probes to latex beads

Chemicals used for buffers were purchased from Sigma-Aldrich, Vienna, Austria. All solutions and buffers were prepared using sterile distilled water and sterile containers, and were filtered through a 0.2 μm acetate filter (Nalgene, VWR International GmbH, Vienna, Austria) into sterile containers before use. All reactions were carried out at ambient temperature. Carboxylate-modified latex beads with approximate diameters of 0.2–0.3 μm and different colors were purchased from Serva Electrophoresis GmbH (Heidelberg, Germany). The parking area (i. e., the available footprint for a carboxyl group on the microsphere surface) was between 47 and 57 $Å^2$/COOH. For standard covalent coupling of amino-modified oligonucleotides to carboxylate-modified beads, the following two-step protocol was used: first, to achieve a monodisperse suspension, the beads were briefly sonicated in an ultrasonic water bath (model 1510, Branson Ultrasonics B.V., Soest, The Netherlands). Then, the

Table 1
Oligonucleotide sequences used

Name	Function/target gene	Sequence	PCR fragment
SAL-f1	Forward primer/*invA*	5′-AACAGTGCTCGTTTACGACC-3′	107 bp
SAL-r1	Reverse primer/*invA*	5′-GCGATCAGGAAATCAACCAG-3′	
SAL-CPL1	Capture probe/*invA*	5′-NH_2-C6-CTGATTCTGGTACTAATG-3′	
SAL-CPL2	Capture probe/*invA*	5′-CATTTCTATGTTCGTCATTCCATTAC-C6-NH_2-3′	
HUM-f1	Forward primer/KCNJ9	5′-TACGTGGAGAAGGATGGCCG-3′	130 bp
HUM-r1	Reverse primer/KCNJ9	5′-CCAGGACGAAGAACAACAGGCT-3′	
HUM-CPL1	Capture probe/KCNJ9	5′-NH_2-C6-GCGCCACTGCAGGTCCACCA-3′	
HUM-CPL2	Capture probe/KCNJ9	5′-GAACAGGTCCGTCAGGTA-C6-NH_2-3′	
HUM-CPL3	Capture probe/KCNJ9	5′-NH_2-C6-GTATGTCTCGCGCACGTT-3′	
HUM-CPL4	Capture probe/KCNJ9	5′-GCCCTGCTGCACGTTG-C6-NH_2-3′	
CON target	Control/synthetic	5′-TTGCATAGCGGGCTTTACGTCCAAGTCGATCAGTAACAAAGTCCAGTCAGT CGAAAAGGGTCGTAGCTCGTAATGCAGAGAGTGTCGATAGTGTG-3′	
CON-f1	Forward primer/synthetic	5′-TTGCATAGCGGGCTTTACGTCCA-3′	95 bp
CON-r1	Reverse primer/synthetic	5′-CACACTATCGACACTCTCTGCATT-3′	
CON-CPL1	Capture probe/synthetic	5′-NH_2-C6-TTTTTTTTTTTTTACGAGCTACGACCCTTTTC-3′	
CON-CPL2	Capture probe/synthetic	5′-GACTGACTGGACTTTGTTTTTTTTTTTTTT-C6-NH_2-3′	