Detection of potential microbial antigens by immuno-PCR (PCR-amplified immunoassay)

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Immu-PCR (PCR-amplified immunoassay; I-PCR) is a novel ultrasensitive method combining the versatility of ELISA with the sensitivity of nucleic acid amplification of PCR. The enormous exponential amplification power of PCR in an I-PCR assay leads to at least a $10^2$–$10^4$-fold increase in sensitivity compared with an analogous ELISA. I-PCR has been used to detect many biological molecules such as proto-oncogenes, toxins, cytokines, hormones, and biomarkers for autoimmune and Alzheimer’s diseases, as well as microbial antigens and antibodies, and it can be adapted as a novel diagnostic tool for various infectious and non-infectious diseases. Quantitative real-time I-PCR has the potential to become the most analytically sensitive method for the detection of proteins. The sensitivity and specificity of a real-time I-PCR assay can be enhanced further with the use of magnetic beads and nanoparticles. This review is primarily focused on the detection of potential viral, bacterial and parasitic antigens by I-PCR assay, thus enabling their application for immunological research and for early diagnosis of infectious diseases.

Introduction

Immuno-PCR (I-PCR) combines the versatility of ELISA with the exponential amplification power and sensitivity of PCR, thus leading to an increase in sensitivity compared with an analogous ELISA. I-PCR is basically similar to ELISA (Fig. 1a), which detects an antigen–antibody reaction, but instead of using an enzyme-conjugated antibody, the antibody is labelled with a DNA fragment, which can be amplified by PCR. Although ELISA is the most commonly used method for the detection of antigens, it fails when there is a low concentration of target antigen. PCR is widely used as a routine laboratory technique for the detection of nucleic acid molecules (Niemeyer et al., 2005, 2007; Mehta et al., 2012b). I-PCR was originally developed by Sano et al. (1993) by using a detection antibody coupled to a biotinylated DNA molecule through a streptavidin–protein A chimeric molecule and could detect as few as 580 BSA antigen molecules. However, the limited availability of streptavidin–protein A fusion protein, and the widely varied affinities of protein A with antibodies of various classes and subclasses from different species restricted its immediate applications. Therefore, Zhou et al. (1993) modified I-PCR to ‘universal I-PCR’ in which an antigen (to be detected) is coupled subsequently with an antigen-specific primary antibody, followed by a species-specific biotinylated detection antibody, streptavidin and a biotinylated DNA (Fig. 1b). The resulting procedure retains the sensitivity and specificity of the original I-PCR but can be universally applied without any immunoglobulin source or (sub)class restrictions.

This novel ultrasensitive I-PCR method has been widely explored for the detection of a variety of biological molecules including cytokines, tumour markers, T-cell receptors, angiotensinogen, toxins, hormones, and biomarkers for autoimmune and Alzheimer’s diseases, and it can be adapted as a novel diagnostic tool for the detection of microbial antigens and antibodies (Niemeyer et al., 2005, 2007; Malou & Raoul, 2011; Potučková et al., 2011; Mehta et al., 2012b). I-PCR has been used as a novel diagnostic tool for the detection of microbial antigens and antibodies (Niemeyer et al., 2005, 2007; Mehta et al., 2012b). I-PCR was originally developed by Sano et al. (1993) by using a detection antibody coupled to a biotinylated DNA molecule through a streptavidin–protein A chimeric molecule and could detect as few as 580 BSA antigen molecules. However, the limited availability of streptavidin–protein A fusion protein, and the widely varied affinities of protein A with antibodies of various classes and subclasses from different species restricted its immediate applications. Therefore, Zhou et al. (1993) modified I-PCR to ‘universal I-PCR’ in which an antigen (to be detected) is coupled subsequently with an antigen-specific primary antibody, followed by a species-specific biotinylated detection antibody, streptavidin and a biotinylated DNA (Fig. 1b). The resulting procedure retains the sensitivity and specificity of the original I-PCR but can be universally applied without any immunoglobulin source or (sub)class restrictions.

Several commonly used formats of universal I-PCR include direct I-PCR, indirect I-PCR, sandwich I-PCR and indirect sandwich I-PCR (Fig. 1b). In direct I-PCR, the antigen to be detected is coated on wells of a microtitre plate, and...
biotinylated detection antibody is then added, which is further attached to a biotinylated reporter DNA through streptavidin (Liang et al., 2003; Zhang et al., 2008). In indirect I-PCR, the antigen to be detected is coated on the wells of a microtitre plate and the detection antibody is then added, followed by the addition of biotinylated antibody against the detection antibody (anti-detection antibody), which is attached to biotinylated reporter DNA through streptavidin (Chao et al., 2004). In sandwich I-PCR, the antigen is sandwiched between a capture antibody on the wells of a microtitre plate and a biotinylated detection antibody, which is attached to biotinylated reporter DNA through streptavidin (Chye et al., 2004). In indirect sandwich I-PCR, the antigen is sandwiched between a

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**Fig. 1.** (a) Evolution of I-PCR. The general set-up of I-PCR is almost similar to that of antigen detection with ELISA, but in I-PCR, a conjugate comprising an antibody and a reporter DNA is amplified by PCR for signal generation. (b) Different formats of universal I-PCR. Streptavidin acts as a linker molecule between the biotinylated detection antibody and the biotinylated reporter DNA; the biotinylated reporter DNA is amplified by PCR/real-time PCR. (i) Direct I-PCR: the antigen is captured on the wells of a microtitre plate and detected directly by biotinylated antibody, which is attached to biotinylated DNA through streptavidin. (ii) Indirect I-PCR: a biotinylated antibody against the detection antibody (anti-detection antibody) is attached to the detection antibody, and is further attached to biotinylated DNA through streptavidin. (iii) Sandwich I-PCR: the antigen is sandwiched between a capture antibody and the detection antibody, which is further attached to biotinylated DNA through streptavidin. (iv) Indirect sandwich I-PCR: the antigen is sandwiched between a capture antibody and the detection antibody, and an anti-detection biotinylated antibody is attached to the detection antibody, which is further attached to biotinylated DNA through streptavidin.
capture antibody on the wells of a microtitre plate and a
detection antibody, and the biotinylated anti-detection
antibody is attached to the detection antibody and linked
to biotinylated reporter DNA through streptavidin (Mehta
et al., 2012b). Sandwich I-PCR is advantageous over the
direct I-PCR method for antigen detection as it eliminates
the need for the direct coating of biological samples as a
source of antigen, which may decrease non-specific binding
without compromising its stability (Niemeyer et al., 1997;
Mehta et al., 2012b). Real-time I-PCR has emerged as the
most sensitive assay for the detection of protein targets.
Sims et al. (2000) were the first researchers to use real-time
I-PCR for the detection of vascular endothelial growth
factor within human and mouse sera. In contrast to the
high sensitivities reported from the other laboratories, no
further increase in ELISA sensitivity was observed by these
researchers, probably due to matrix effects attributed to
non-specific binding of serum constituents that increased
the background noise and hence influenced the perform-
ance of the I-PCR. The non-specific binding occurring with
the use of classical microtitre plates (solid format) can
be reduced by using appropriate blocking solutions. The
introduction of a liquid format, for example the use
of magnetic beads/gold nanoparticles (Adler et al., 2008;
Barletta et al., 2009; Perez et al., 2011; Adams et al., 2012),
has been a major breakthrough in devising I-PCR assays, as
it allows more thorough washing of the captured antigen
and nanoparticles to decrease non-specific binding and
reduce the background signals (Malou & Raoult, 2011;
Perez et al., 2011). The large surface area of nanoparticles in
comparison with that of microtitre plates also permits a
higher and a faster interaction between the capture antibody
and respective antigen.

There can be direct conjugation of detection antibody
with the reporter DNA through covalent binding with a
chemical compound such as succinimidyl 4-[N-maleimi-
domethyl]-cyclohexane-1-carboxylate (SMCC) (Wu
et al., 2001; Liang et al., 2003) as shown in Fig. 2(a). This assay
is quicker to perform than a streptavidin–biotin-based I-PCR,
as washing is required only after the addition of sample
antigen and after the addition of the DNA–antibody con-
jugate. Another important strategy is phage display-
mediated I-PCR (PD-I-PCR; Fig. 2b). Instead of using
mAb and streptavidin–biotin/chemically linked DNA in the
conventional I-PCR, a recombinant phage particle is used as
a ‘ready reagent’ for I-PCR (Guo et al., 2006). The surface-
displayed single chain variable fragment (scFv) and phage
DNA themselves can serve directly as detection antibody and
PCR template, respectively. The target antigen is captured
by the immobilized capture antibody coated on a microtitre
plate, whilst the recombinant phage particles are anchored
through the interaction between the displayed scFv and
bound target antigen. The phage DNA is released by heat
lysis and serves as PCR template for amplification. PD-I-
PCR has merits over conventional I-PCR, as the recombin-
ant phage particles carry both scFv and DNA to be amplified
and they can easily be obtained by simply centrifuging
the overnight Escherichia coli culture broth. However, the
sensitivity of PD-I-PCR is not as high as that of mAb-based
I-PCR due to the lower affinity of scFv towards the antigen.

In recent years, nanobiotechnology has emerged as the most
promising tool for the development of a powerful strategy
for drug delivery diagnostics (Griffiths et al., 2010). Besides
circumventing the background noise, the use of magnetic
beads and nanoparticle-based I-PCR can further improve
detection limits and reduce washing steps, as well as
reducing incubation steps, thus improving the assay (Adler
et al., 2008; Chen et al., 2009; Perez et al., 2013). In the
magnetic bead-based I-PCR assay (Fig. 2c), the capture
antibody is adsorbed to the magnetic beads to capture the
antigen (Barletta et al., 2009) and the sandwich I-PCR format
is then followed using streptavidin as a bridge between the
detection antibody and the reporter DNA. Briefly, the
magnetic beads are captured (using a magnetic plate placed
under the microtitre plate) periodically after each washing
phase; the magnetic beads are then released from the
magnetic force and allowed to circulate freely in solution
during incubation with antigen or antibody. Another I-PCR
format is gold nanoparticle I-PCR (GNP-I-PCR; Fig. 2d).
The antigen is captured by the specific polyclonal antibody
coated on the wells of a microtitre plate, followed by the
addition of gold nanoparticle (GNP) probes [functionalized
with thiolated oligonucleotides (capture DNA) and mAb] to
form sandwich immunocomplexes (Chen et al., 2009). The
oligonucleotides on the GNP contain two strands: one as a
capture DNA immobilized on the surface of the GNP and the
other as a signal DNA, which is partially complementary to
the capture DNA. The immunocomplexes are heated to
release the signal DNA, which is quantified by real-time PCR.
In another I-PCR format, known as nanoparticle-amplified
I-PCR (NPA-I-PCR) (Fig. 2e), antigen is captured using
antibody-functionalized magnetic beads (Perez et al., 2011,
2013). The gold nanoparticles are functionalized with the
detection antibodies and thiolated DNA complementary
to the hybridized tag DNA. The magnetic bead–antigen
complex is reacted with antibody–DNA-functionalized
GNPs. The hybridized tag DNA (signal DNA) is released
from the GNPs by heating and quantified by real-time PCR.

I-PCR assay is not restricted to proteins: many small molecular
targets are also detected up to femtogram levels; for example,
biogenic amine serotonin has been detected by sandwich
I-PCR with a 1000-fold increase in sensitivity compared with
an analogous ELISA (Adler et al., 2005). In addition, the assay
has been translated into a commercial kit (Chimera Biotech;
http://www.chimera-biotech.com) (Wacker, 2006), thus
demonstrating that I-PCR is progressing from the research and
development platform to a routine diagnostic method.
Recently, Malou & Raoult (2011) and previously Niemeyer
et al. (2005, 2007) meticulously reviewed the detection of
various antigens and antibodies using an ultrasensitive I-PCR
assay. In this review, we have further updated our knowledge
of the detection of potential viral, bacterial and parasitic
antigens by I-PCR assay, and its application in immunological
research and clinical diagnosis of infectious diseases.
Detection of viral antigens

Although PCR is widely employed to detect nucleic acid molecules in viral infections, proteins remain the major pathological mediators and are detected primarily by ELISA. In recent years, I-PCR assays have been exploited for the detection of viral protein molecules. Maia et al. (1995) were
the first researchers to develop a sandwich I-PCR assay for the detection of viral antigens, including the hepatitis B surface antigen (HBsAg) of hepatitis B virus (HBV), which combined the specificity of two high-affinity anti-HBsAg mAbs employed both as capture and as detection antibody (Table 1). The use of two-site mAbs was found to greatly enhance the specificity and sensitivity of the assay such that it could detect as little as 0.5 pg HBsAg in serum samples (Maia et al., 1995).

Several laboratories have demonstrated high-sensitivity detection (100–700-fold increase) of HBsAg by I-PCR compared with ELISA (Niemeyer et al., 1997; Cao et al., 2000). Magneto I-PCR was devised by Wacker et al. (2007) for the better detection of recombinant HBsAg in human serum. The HBsAg-specific antibody–magnosome conjugate and the DNA–antibody conjugate were incubated simultaneously with a serum sample containing HBsAg resulting in a signal-generating detection complex. The detection complex was concentrated using an external magnetic field to quantify the immobilized HBsAg by real-time PCR with a detection limit of 320 pg ml⁻¹ compared with 40 ng ml⁻¹ detected by Magneto ELISA. As well as HBsAg, the core antigen of HBV (HBcAg) is another potential biomarker for the identification of HBV infection. Recently, a TaqMan real-time PCR assay based on PD-I-PCR has been developed for the detection of HBcAg in human serum samples (Monjezi et al., 2013). In this assay, a constrained peptide displayed on the surface of an M13 recombinant bacteriophage that interacted tightly with HBcAg was employed as a diagnostic reagent and could detect as little as 10 ng HBcAg with 10⁸ p.Eu. M13 recombinant phage ml⁻¹, which was about 10 000 times more sensitive than the phage ELISA.

Because of the low concentration of p24 antigen in the early stage of human immunodeficiency virus (HIV) infection, these viral proteins in particular have become a primary target in I-PCR developments (Barletta et al., 2004, 2009). Detection of HIV-1 p24 antigen increases the probability of HIV-1 detection, as free HIV-1 p24 antigen circulates in the blood and/or is sequestered in immune complexes and tissues in the absence of virions (Barletta et al., 2004). Barletta et al. (2004) documented a quantitative real-time I-PCR assay to determine HIV-1 infection by detecting the presence of HIV-1 p24 antigen in serum samples with an elevated sensitivity in comparison with RNA detection by reverse transcription-PCR (RT-PCR), which had a sensitivity of 50 viral copies ml⁻¹. The HIV-1 p24 antigen detection was more sensitive for the detection of virus, as this target protein is present in the virion at much higher numbers than the viral RNA copies (approx. 3000 HIV-1 p24 antigen molecules versus two RNA copies per virion). Of 52 samples of HIV-1-infected patients (with <50 RNA copies ml⁻¹), 22 samples were found to be positive by real-time I-PCR. Later, a modified quantitative real-time I-PCR assay based on magnetic beads was devised by Barletta et al. (2009). The HIV-1 p24 antigen was captured by an anti-HIV-1 p24 antibody adsorbed to the magnetic beads, which were captured by a magnetic plate. The p24 antigen was then detected by a biotinylated anti-p24 antibody linked to a biotinylated reporter DNA via streptavidin–horseradish peroxidase. The use of streptavidin–horseradish peroxidase allowed ELISA to be performed simultaneously, as well as the amplification and quantification of the reporter DNA. This assay had a limit of quantification of 10–100 p24 antigen molecules, enabling a detection limit of less than one HIV-1 virion in a complex sample matrix (plasma) and providing better potential to monitor an early HIV-1 infection than detecting the nucleic acid molecules by PCR (Barletta et al., 2009). The ultralow level of detection of p24 antigen by I-PCR is not just of academic interest but might also have potential implications in examining HIV pathogenesis and anti-retroviral treatment strategies. Standardization and optimization of this method are needed to move the method to a framework that would make it a candidate for routine use in clinical diagnosis.

Recently, an immunoliposome-PCR (IL-PCR) assay was introduced to quantify recombinant HIV-1 p24 antigen in buffer and carcinoembryonic antigen in human serum with a detection limit of 2.4 pg ml⁻¹ for p24 antigen (He et al., 2012). This method used a liposome preparation incorporating reporter DNA encapsulated inside and a biotin-labelled polyethylene glycol phospholipid conjugate as a detection reagent in the outer surface of a liposome, which is coupled to real-time PCR for antigen detection. This assay has a number of merits over other I-PCR formats, as the reporter DNA and detection reagent are incorporated into the liposomes, thus simplifying preparation of the detection reagent. The ability to encapsulate multiple reporters per liposome could also overcome the effect of polymerase inhibitors present in many biological specimens and thus might have wide applications for the diagnosis of infectious diseases.

Rotavirus infection causes serious diarrhoeal disease in children at an early stage of their life. To minimize the risk of rotavirus infection, a highly sensitive quantitative real-time I-PCR was developed by Adler et al. (2005) for early detection of the rotavirus antigen VP6. The VP6 antigen was immobilized between mouse anti-rotavirus mAb and CHI-Rota (a chimaeric conjugate of mouse anti-rotavirus mAb and marker DNA) to attain a detection limit of 100 virus particles ml⁻¹ by quantitative real-time I-PCR assay using standardized I-PCR protocols (Imperacer; Chimera Biotec). Similar results were also obtained with a PCR-ELISA, in which the amplified products were labelled with biotin as well as with DIG, and later the labelled amplified products were attached to a streptavidin-coated microtitre plate for quantification using anti-DIG–alkaline phosphatase antibody–enzyme conjugate and fluorescence-generating substrate (Adler et al., 2005).

Recently, Perez et al. (2011, 2013) described an NPA-I-PCR assay that combined traditional ELISA with a 50-fold nanoparticle valence amplified step, followed by real-time PCR amplification. The assay could detect respiratory
Table 1. Sensitivities of I-PCR assays with various microbial antigens

<table>
<thead>
<tr>
<th>Type of microbe antigen</th>
<th>Format of I-PCR</th>
<th>Target antigen</th>
<th>LOD</th>
<th>Reference</th>
<th>Brief description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral antigens</td>
<td>Sandwich I-PCR</td>
<td>HBsAg</td>
<td>0.5 pg per sample</td>
<td>Maia et al. (1995)</td>
<td>10^2-fold more sensitive than radioimmunoassay</td>
</tr>
<tr>
<td></td>
<td>Indirect I-PCR</td>
<td>HBV-1 antigen</td>
<td>ND</td>
<td>Mweene et al. (1996)</td>
<td>Up to 10^7-fold more sensitive than ELISA or PCR</td>
</tr>
<tr>
<td></td>
<td>Indirect I-PCR</td>
<td>NSP-1 of equine influenza virus</td>
<td>1.9 × 10^4 p.f.u. ml^-1</td>
<td>Ozaki et al. (2001)</td>
<td>10^2-fold more sensitive than RT-PCR</td>
</tr>
<tr>
<td></td>
<td>Sandwich real-time I-PCR</td>
<td>HIV-1 p24 antigen</td>
<td>4 × 10^-5 pg per reaction</td>
<td>Barletta et al. (2004)</td>
<td>25-fold more sensitive than RT-PCR</td>
</tr>
<tr>
<td></td>
<td>Sandwich real-time I-PCR (Imperacer)</td>
<td>Rotavirus antigen</td>
<td>100 virus particle ml^-1</td>
<td>Adler et al. (2005)</td>
<td>10^2-fold more sensitive than ELISA</td>
</tr>
<tr>
<td></td>
<td>PD-I-PCR</td>
<td>HNP antigen</td>
<td>1 × 10^6 pg ml^-1</td>
<td>Guo et al. (2006)</td>
<td>10^2-10^4-fold more sensitive than ELISA</td>
</tr>
<tr>
<td></td>
<td>Indirect sandwich real-time I-PCR</td>
<td>NV capsid protein</td>
<td>6.6 × 10^2 equivalent particle units</td>
<td>Tian &amp; Mandrell (2006)</td>
<td>Sensitivity &gt;10^5-fold higher than ELISA and 10-fold higher than RT-PCR</td>
</tr>
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<td></td>
<td>Magneto I-PCR</td>
<td>HBsAg</td>
<td>320 pg ml^-1</td>
<td>Wacker et al. (2007)</td>
<td>10^2-fold more sensitive than Magneto ELISA</td>
</tr>
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<td></td>
<td>Magnetic bead-based real-time I-PCR</td>
<td>HIV-1 p24 antigen</td>
<td>&lt;100 molecules per reaction</td>
<td>Barletta et al. (2009)</td>
<td>Could detect &lt;1 HIV-1 virion in a complex sample matrix (plasma)</td>
</tr>
<tr>
<td></td>
<td>GNP-IPCR</td>
<td>HNP antigen</td>
<td>1 × 10^-2 pg ml^-1</td>
<td>Chen et al. (2009)</td>
<td>Approx. seven orders of magnitude more sensitive than ELISA</td>
</tr>
<tr>
<td></td>
<td>NPA-I-PCR</td>
<td>RSV surface protein</td>
<td>4.1 p.f.u. ml^-1</td>
<td>Perez et al. (2011)</td>
<td>4 × 10^5-fold more sensitive than ELISA and fourfold more sensitive than RT-PCR</td>
</tr>
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<td></td>
<td>GNP-I-PCR (conventional PCR)</td>
<td>Purified FMDV</td>
<td>1 × 10^-2 pg ml^-1</td>
<td>Ding et al. (2011)</td>
<td>10^2-fold more sensitive than ELISA</td>
</tr>
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<td>Real-time I-PCR (chemical conjugation of detection antibody and DNA)</td>
<td>H5N1 AIV</td>
<td>10^-4 EID_{50} ml^-1</td>
<td>Deng et al. (2011a)</td>
<td>10^1-fold more sensitive than ELISA and 10^2-fold more sensitive than RT-PCR</td>
</tr>
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<td></td>
<td>Magnetic bead-based sandwich I-PCR</td>
<td>H5N1AIV and NDV</td>
<td>10^-4 EID_{50} ml^-1</td>
<td>Deng et al. (2011b)</td>
<td>Could detect trace levels of H5N1AIV and NDV in one step</td>
</tr>
<tr>
<td></td>
<td>Sandwich IL-PCR</td>
<td>HIV-1 p24 antigen</td>
<td>2.4 pg ml^-1</td>
<td>He et al. (2012)</td>
<td>Reporter DNA and detection reagent were incorporated into liposomes, simplifying preparation of the detection reagent</td>
</tr>
<tr>
<td>Prion proteins</td>
<td>PD-I-PCR</td>
<td>HBcAg</td>
<td>1 × 10^5 pg</td>
<td>Monjezi et al. (2013)</td>
<td>10^4-fold more sensitive than phage ELISA</td>
</tr>
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<td></td>
<td>Sandwich real-time I-PCR</td>
<td>rNV-VLP</td>
<td>&gt;10^5 VLPs ml^-1</td>
<td>Matsushita et al. (2013)</td>
<td>&gt;10^5-fold sensitive than ELISA</td>
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<td>Sandwich real-time I-PCR</td>
<td>Bovine PrP</td>
<td>7.5 × 10^2 pg ml^-1</td>
<td>Gofflot et al. (2004)</td>
<td>10-fold sensitive than ELISA</td>
</tr>
<tr>
<td></td>
<td>Sandwich real-time I-PCR</td>
<td>Human brain PrP</td>
<td>1 × 10^5 pg ml^-1</td>
<td>Gofflot et al. (2005)</td>
<td>&gt;10-fold sensitive than ELISA</td>
</tr>
<tr>
<td></td>
<td>Sandwich real-time I-PCR</td>
<td>Hamster brain PrP</td>
<td>1.9 × 10^-2 pg ml^-1</td>
<td>Barletta et al. (2005)</td>
<td>10^6-fold more sensitive than ELISA or Western blot</td>
</tr>
<tr>
<td></td>
<td>Sandwich real-time I-PCR</td>
<td>Hamster brain PrP^2</td>
<td>1 × 10^-3 pg ml^-1</td>
<td>Guo et al. (2006)</td>
<td>10^2-10^4-fold more sensitive than ELISA</td>
</tr>
</tbody>
</table>
Table 1. cont.

<table>
<thead>
<tr>
<th>Type of microbe antigen</th>
<th>Format of I-PCR</th>
<th>Target antigen</th>
<th>LOD</th>
<th>Reference</th>
<th>Brief description</th>
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<tr>
<td>Bacterial antigens</td>
<td>Indirect I-PCR</td>
<td><em>P. piscidia</em></td>
<td>3.4 c.f.u. ml⁻¹</td>
<td>Kakizaki et al. (1996)</td>
<td>10⁴-fold more sensitive than ELISA</td>
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<td>Sandwich I-PCR</td>
<td><em>E. coli</em> β-glucuronidase</td>
<td>1 x 10⁻³ pg ml⁻¹</td>
<td>Chang &amp; Huang (1997)</td>
<td>10²-fold more sensitive than enzyme-capture assay</td>
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<td>Modified I-PCR (Indirect I-PCR + in situ I-PCR)</td>
<td>LPS of <em>Bacteroides fragilis</em></td>
<td>10⁴ c.f.u. ml⁻¹</td>
<td>Zhang et al. (1998)</td>
<td>Performed on <em>in situ</em> PCR slides to localize the detected antigen</td>
<td></td>
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<tr>
<td>Direct I-PCR (chemical conjugation of detection antibody and DNA)</td>
<td>BoNT/A</td>
<td>5 pg</td>
<td>Wu et al. (2001)</td>
<td>10³-fold more sensitive than ELISA</td>
<td></td>
</tr>
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<td>Direct I-PCR</td>
<td><em>Streptococcus</em> pyogenes group A</td>
<td>10⁻³ bacterial cells per well</td>
<td>Liang et al. (2003)</td>
<td>Direct I-PCR method was 10⁴-fold more sensitive than I-PCR (chemical conjugation of detection antibody and DNA) method</td>
<td></td>
</tr>
<tr>
<td>I-PCR (chemical conjugation of detection antibody and DNA)</td>
<td><em>Streptococcus</em> pyogenes group A</td>
<td>10 bacterial cells per well</td>
<td>Chao et al. (2004)</td>
<td>10²-fold more sensitive than ELISA</td>
<td></td>
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<tr>
<td>Indirect I-PCR and indirect sandwich I-PCR</td>
<td>BoNT/A</td>
<td>5 x 10⁻² pg ml⁻¹</td>
<td>Huang &amp; Chang (2004)</td>
<td>Could detect 144 antigen molecules ml⁻¹</td>
<td></td>
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<td>Sandwich I-PCR</td>
<td>Protein A of <em>Staphylococcus aureus</em></td>
<td>10⁻⁵ pg ml⁻¹</td>
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<td>Sandwich real-time I-PCR</td>
<td>SEB</td>
<td>&lt;10 pg ml⁻¹</td>
<td>Rajkovic et al. (2006)</td>
<td>10³-fold more sensitive than ELISA and a detection range of 10 to 30,000 pg ml⁻¹</td>
<td></td>
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<td>Direct I-PCR (chemical conjugation of detection antibody and DNA; microtitre plates)</td>
<td><em>Cry1Ac</em> protein of <em>B. thuringiensis</em></td>
<td>21.6 x 10⁻³ pg ml⁻¹</td>
<td>Allen et al. (2006)</td>
<td>The microtitre plate assay was more sensitive than streptavidin particle-based assay</td>
<td></td>
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<tr>
<td>Direct I-PCR (chemical conjugation of detection antibody and DNA; streptavidin-coated particles)</td>
<td><em>Cry1Ac</em> protein of <em>B. thuringiensis</em></td>
<td>43.2 x 10⁴ pg toxin ml⁻¹</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Indirect sandwich real-time I-PCR</td>
<td>SEA and SEB</td>
<td>SEA, 6 pg per well; SEB, 0.6 pg per well</td>
<td>Fischer et al. (2007)</td>
<td>10¹⁻¹⁰²-fold more sensitive than EIA</td>
<td></td>
</tr>
<tr>
<td>Direct I-PCR</td>
<td>9a5c bacterial strain of <em>X. fastidiosa</em></td>
<td>10 bacterial cells</td>
<td>Peroni et al. (2008)</td>
<td>10⁴⁻¹⁰⁶-fold more sensitive than ELISA and PCR; 10³-fold more sensitive than IC-PCR assay</td>
<td></td>
</tr>
<tr>
<td>Direct I-PCR</td>
<td>Stx2 from STEC</td>
<td>10 pg ml⁻¹</td>
<td>Zhang et al. (2008)</td>
<td>10²-fold more sensitive than EIA</td>
<td></td>
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<tr>
<td>Sandwich real-time I-PCR</td>
<td>Stx2 from STEC</td>
<td>0.1 pg ml⁻¹</td>
<td>He et al. (2011)</td>
<td>10²-fold more sensitive than ELISA, with a detection range of 10 to 100,000 pg ml⁻¹</td>
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<tr>
<td>Indirect real-time I-PCR</td>
<td><em>Y. pestis</em> in dental pulp of ancient specimens</td>
<td>0.74 x 10⁻³ pg ml⁻¹</td>
<td>Malou et al. (2012b)</td>
<td>70-fold more sensitive than ELISA</td>
<td></td>
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<tr>
<td>Indirect sandwich I-PCR</td>
<td>ESAT-6, CFP-10, CFP-21 and MPT-64 antigens of <em>M. tuberculosis</em></td>
<td>1 x 10⁻⁴ pg ml⁻¹</td>
<td>Mehta et al. (2012b)</td>
<td>10⁷-fold more sensitive than ELISA</td>
<td></td>
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<tr>
<td>Sandwich real-time I-PCR</td>
<td>Stx2 from STEC</td>
<td>0.01 pg ml⁻¹</td>
<td>He et al. (2013)</td>
<td>10²-fold more sensitive than ELISA</td>
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</table>
syncytial virus surface protein in paediatric infection using antibody-functionalized magnetic beads and a Synagis antibody bound to a GNP co-functionalized with thiolated DNA. This assay showed a 4000-fold improvement in the limit of detection (LOD) compared with ELISA and a fourfold improvement in the LOD compared with RT-PCR from HEp-2 cell extracts, suggesting that the NPA/I-PCR assay could offer a viable platform for the development of early-stage diagnostics requiring exceptionally low levels of detection.

Norovirus (NV) is an important human pathogen that causes epidemic acute non-bacterial gastroenteritis on a global scale (Glass et al., 2001). A sensitive real-time I-PCR method was devised by Tian & Mandrell (2006) for the detection of NV capsid protein in stool and food samples, which showed an improved LOD compared with ELISA and RT-PCR assays. The removal of micro-organisms by drinking-water treatment processes has been examined in laboratory-scale experiments using artificially propagated micro-organisms; however, this method cannot be applied to NV removal because of the lack of a cell-culture system or an animal model for this virus. This fact has restricted the ability to examine its behaviour during drinking-water treatment. To overcome this problem, Shirasaki et al. (2010) previously used recombinant NV virus-like particles (rNV-VLPs), which comprised an artificially expressed NV capsid protein, in laboratory-scale drinking-water treatment experiments. The ELISA method used to detect rNV-VLPs was not sensitive enough to examine high removal ratios such as those obtained by ultrafiltration. A highly sensitive real-time I-PCR assay has been described for the quantification of rNV-VLPs to investigate NV removal by microfiltration, ultrafiltration and hybrid pre-coagulation–microfiltration processes (Matsushita et al., 2013).

Mweene et al. (1996) earlier introduced an I-PCR assay for avian influenza virus, which could detect an ultralow concentration of avian influenza virus in clinical samples of allantoic fluid from eggs and treated swab specimens in the poultry. This assay could detect up to $10^{4}$ 50% egg infectious doses from eggs and treated swab specimens in the poultry.

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Table 1. cont.

<table>
<thead>
<tr>
<th>Type of microbe antigen</th>
<th>Format of I-PCR</th>
<th>Target antigen</th>
<th>LOD</th>
<th>Reference</th>
<th>Brief description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parasitic antigen</td>
<td>Sandwich I-PCR</td>
<td>204 kDa Acl₅ of A. cantonensis (fifth-stage larvae)</td>
<td>100 pg ml⁻¹</td>
<td>Chye et al. (2004)</td>
<td>10⁻⁴–10⁻³-fold more sensitive than ELISA</td>
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</table>

HRAg, hepatitis B virus surface antigen; BHV-1, bovine herpes virus-1; ND, not determined; NSP-1, non-structural protein-1; RT-PCR, reverse transcription PCR; HIV, human immunodeficiency virus; HNP, Hantaan virus nucleocapsid protein; NV, norovirus; RSV, respiratory syncytial virus; FMDV, foot-and-mouth disease virus; AIV, avian influenza virus; EID₅₀, 50% egg infectious dose; NDV, Newcastle disease virus; IL-PCR, immunoliposome-PCR; HBeAg, core antigen of hepatitis B virus; rNV-VLP, recombinant norovirus-virus-like particles; PrP, prion protein; PrPSc, cellular prion protein; PrPSc, prion protein scrapie; BoNT/A, C. botulinum neurotoxin type A; SEA, Staphylococcus aureus enterotoxin A; SEB, Staphylococcus aureus enterotoxin B; EIA, enzyme immunoassay; IC-PCR, immunocapture PCR; ESAT-6, early secretory antigenic target-6; CFP-10, culture filtrate protein-10; CFP-21, culture filtrate protein-21; MPT-64, mycobacterial protein from species tuberculosis-64.
(EID\textsubscript{50}) ml\textsuperscript{-1} of avian influenza virus using SMCC as a cross-linker between the reporter DNA and the detecting antibody. In addition, the reporter DNA was cut with BamHI and quantified by real-time I-PCR, thus enabling an improved detection by at least 10\textsuperscript{2}-fold in comparison with RT-PCR and a 10\textsuperscript{3}-fold improvement in comparison with ELISA. Later, Deng et al. (2011) introduced an I-PCR assay utilizing magnetic particles as the adsorption surface and adopting the conventional method of streptavidin linking the detection antibody with the reporter DNA for the detection of both avian influenza virus and Newcastle disease virus in one step. A detection limit of as low as 10\textsuperscript{-4} EID\textsubscript{50} ml\textsuperscript{-1} was observed for both the viruses, thus providing a viable scaffold capable of mass screening as well as detection of these infections in one step.

The RNA-binding nucleoprotein has been shown to be an excellent target for the development of influenza A virus diagnostics due to its high antigenicity and also its presence in large numbers in the virus particles. Interestingly, it binds non-specifically to the sugar–phosphate backbone of RNA as well as to ssDNA in an \textit{in vitro} system. Recently, Morin & Schaeffer (2012) took advantage of this property to develop an ssDNA probe for the detection of recombinant influenza A virus nucleoprotein in the low picomolar range using a quantitative PCR assay.

Foot-and-mouth disease virus (FMDV) causes contagious infection in artiodactyls, especially in cattle and swine, leading to huge economic losses (Ding et al., 2011). ELISA and conventional PCR are the standard laboratory tests to detect FMDV; however, these tests have their limitations. A highly sensitive GNP-I-PCR assay derived from the bio-barcode assay has been developed for the detection of FMDV (Nam et al., 2003; Ding et al., 2011). The viral particles were sandwiched between the polyclonal antibody and an FMDV-specific mAb (clone 1D11) attached to GNP along with the surface-bound oligonucleotides to form an immune complex. The signal DNA was released by heating and consequently evaluated by PCR/real-time PCR. The detection limit of the GNP-I-PCR assay could reach up to 10 fg ml\textsuperscript{-1} for purified FMDV particles in comparison with a detection limit of 100 ng ml\textsuperscript{-1} using ELISA. A modified form of bio-barcode assay has also been devised for the ultrasensitive detection of Hantaan virus nucleocapsid protein by GNP-I-PCR assay and could determine up to 10 fg ml\textsuperscript{-1} of the purified nucleocapsid protein in buffers as well as in human serum samples (Chen et al., 2009).

Detection of prion proteins (PrPs)

PrPs are known to cause fatal neurological disorders (Gofflot et al., 2004, 2005). The cellular prion proteins (PrP\textsuperscript{Sc}) are modified to pathogenic PrP by conformational changes of \(\alpha\)-helices into \(\beta\)-sheets resulting in the formation of proteinase K-resistant PrP forms; this forms the basis for the diagnosis of prion infections. The introduction of I-PCR has been a major breakthrough for the detection of PrP. A sandwich real-time I-PCR assay has been demonstrated for the detection of recombinant bovine PrP (Gofflot et al., 2004), and revealed at least a 10-fold higher sensitivity than ELISA. Later, Gofflot et al. (2005) diagnosed PrP in humans infected with sporadic Creutzfeldt–Jakob disease by ELISA, Western blotting, immunohistochemistry and quantitative real-time I-PCR methods. All these methods revealed 100% sensitivity and 100% specificity; however, real-time I-PCR detected the presence of PrP at concentrations at least 10-fold lower than ELISA. Pathological prion protein scrapie (PrP\textsuperscript{Sc}), implicated in transmissible spongiform encephalopathies, is detected primarily by Western blot or ELISA to screen the brainstem in cattle. Using the I-PCR assay, recombinant hamster PrP\textsuperscript{Sc} could be detected consistently at 1 fg ml\textsuperscript{-1}, and proteinase K-digested scrapie-infected hamster brain homogenates diluted to \(10^{-8}\) (approx. 10–100 infectious units) were detected with a semi-quantitative dose–response (Barletta et al., 2005). The level of detection was 1 million-fold more sensitive than the levels detected by Western blot or ELISA, and these researchers recommended I-PCR as a method capable of detecting PrP\textsuperscript{Sc} in the preclinical phase of infection. Furthermore, their findings suggested that, unless complete proteinase K digestion of PrP\textsuperscript{Sc} in biological materials is verified, an ultrasensitive I-PCR assay might inaccurately classify a sample as positive. A sensitive PD-I-PCR assay has also been documented for the detection of PrP as well as purified Hantaan virus nucleocapsid protein by indirect, sandwich and real-time PD-I-PCR formats with enhanced detection sensitivity compared with PD-ELISA (Guo et al., 2006).

As explained above, background signals are a critical factor in I-PCR assays, particularly when using complex protein suspensions like PrP. Recently, Kuczius et al. (2012) were able to reduce background noise by including two heating steps, the first for protein denaturation and the second for detachment of immunocomplexed DNA, enabling optimal DNA amplification with an increased detection sensitivity for the simultaneous detection of PrP\textsuperscript{Sc} and central nervous system indicators.

Detection of bacterial antigens

Rapid and accurate diagnostic tests are required by the clinical laboratories for early diagnosis of bacterial infections, especially for slow-growing and fastidious bacteria that are difficult to isolate. In recent years, I-PCR assays have been reasonably exploited to detect important bacterial antigens.

Kakizaki \textit{et al.} (1996) earlier demonstrated an indirect I-PCR assay for the detection of the fish pathogen \textit{Pasteurella piscicida} in naturally infected yellowtail. The assay detected 3.4 c.f.u. ml\textsuperscript{-1} compared with 3.4 \times 10\textsuperscript{4} c.f.u. ml\textsuperscript{-1} detected with ELISA.

\textit{E. coli} \(\beta\)-glucuronidase is a specific marker used to identify \textit{E. coli} in a variety of diverse samples such as urine and food. Chang & Huang (1997) documented sandwich I-PCR for the detection of glucuronidase with an LOD of...
1 × 10^{-17} \text{ g ml}^{-1} \) (equivalent to 40 glucuronidase molecules ml\(^{-1}\)), which was 10^8 times more sensitive than ELISA. The utility of I-PCR method as a generic strategy for rapid screening of fruit juices and plant products for the detection of enterohaemorrhagic \textit{E. coli} O157: H7 has also been demonstrated (Ogunjimi & Choudary, 1999).

Zhang et al. (1998) developed a modified I-PCR assay, which was a combination of indirect I-PCR and \textit{in situ} I-PCR for the detection of \textit{Bacteroides fragilis} using a mAb that recognizes a specific epitope in the LPS present in \textit{Bacteroides fragilis}. This method was a combination of indirect I-PCR and \textit{in situ} I-PCR formats. Using the same system, as these are members of the family of pyrogenic enterotoxins (Rajkovic \textit{et al.}, 2006; Fischer \textit{et al.}, 2011). STEC serotypes in the environment have been reported frequently, but detection of STEC in these environmental samples remains difficult because the numbers of bacteria are often below the detection threshold of the methods employed. Enzyme immunoassay (EIA) fails to detect many of the Stx2 strains because of the production of Stx2 below the level of detection (Zhang \textit{et al.}, 2005). A direct I-PCR was developed by Zhang \textit{et al.} (2008) to detect purified Stx2 with enhanced detection sensitivity. Moreover, this assay could detect Stx2 and variants in STEC strains that produce the toxins at levels that are non-detectable by EIA and in EIA-negative enriched stool cultures from patients. He \textit{et al.} (2011) demonstrated a real-time I-PCR assay for the detection of Stx2 and variants in environmental samples contaminated with soil, faeces and water samples, whilst ELISA could not detect Stx2 in these contaminated samples. The quantitative detection limit of

**Streptococcus pyogenes** (also known as group A \textit{Streptococcus}) is associated with a number of infections in humans, including acute pharyngitis, pneumonia, sepsis, acute rheumatic fever and post-streptococcal glomerulonephritis (Musser \textit{et al.}, 1995). For the detection of \textit{Streptococcus pyogenes}, two I-PCR formats were exploited by Liang \textit{et al.} (2003). In the first format, streptavidin was used as a linker molecule between the biotinylated reporter DNA and the biotinylated anti-group A \textit{Streptococcus} mAb with an LOD of 10^{-5} bacterial cells per well, which was much lower than commercial Strep A test kits with LOD ranges of 10^2–5 × 10^5 bacterial cells per test. In the second format, there was a covalent conjugation of the anti-group A mAb with the reporter DNA through SMCC with an LOD of 10 cells per well.

The Shiga toxin (Stx)-producing \textit{E. coli} (STEC) serotype O157 : H7 and several non-O157 : H7 serotypes have emerged worldwide, and cause diarrhoea, haemorrhagic colitis and the life-threatening haemolytic–uraemic syndrome in humans (Zhang \textit{et al.}, 2008; He \textit{et al.}, 2011). STEC serotypes in the environment have been reported frequently, but detection of STEC in these environmental samples remains difficult because the numbers of bacteria are often below the detection threshold of the methods employed. Enzyme immunoassay (EIA) fails to detect many of the Stx2 strains because of the production of Stx2 below the level of detection (Zhang \textit{et al.}, 2005). A direct I-PCR was developed by Zhang \textit{et al.} (2008) to detect purified Stx2 with enhanced detection sensitivity. Moreover, this assay could detect Stx2 and variants in STEC strains that produce the toxins at levels that are non-detectable by EIA and in EIA-negative enriched stool cultures from patients. He \textit{et al.} (2011) demonstrated a real-time I-PCR assay for the detection of Stx2 and variants in environmental samples contaminated with soil, faeces and water samples, whilst ELISA could not detect Stx2 in these contaminated samples. The quantitative detection limit of

**Bacillus thuringiensis** \textit{var. kurstaki} serves as an important reservoir of Cry1Ac toxin protein for the production of biological insecticides and insect-resistant genetically modified crops (Romeis \textit{et al.}, 2006). The gene encoding this toxin has also been genetically transformed into crop plants. A reliable method for detecting the Cry1Ac toxin would facilitate monitoring for this protein in crop and non-crop plants, as well as in foods from the transgenic plants. Allen \textit{et al.} (2006) introduced an I-PCR assay for the detection of purified Cry1Ac protein in which a polyclonal antibody was used as the detection antibody, which was covalently coupled to a reporter DNA through SMCC on two different surfaces: (i) polyvinyl chloride microtitre plates and (ii) streptavidin-coated particles. In the polyvinyl chloride microtitre plate, a direct I-PCR format was followed, whilst in the second method, Cry1Ac protein was conjugated directly onto the streptavidin-coated particles and an I-PCR assay was then performed. Interestingly, the sensitivity of the polyvinyl chloride microtitre plate for the detection of Cry1Ac protein was found to be higher than the streptavidin-coated particles.

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the assay was 0.1 pg ml⁻¹ in buffer with a quantification range of 10–100 000 pg ml⁻¹, which was 10 000-fold more sensitive than ELISA. Furthermore, there was direct attachment of streptavidin-conjugated detection antibody to the biotinylated DNA rather than using streptavidin as a bridge between the biotinylated detection antibody and the biotinylated DNA, which also facilitated reducing the non-specific signal.

Although outbreaks of haemolytic–uraemic syndrome due to the consumption of dairy products occur frequently, few reports are available on assays for the detection of Stx2 in milk. A highly sensitive real-time I-PCR assay capable of detecting 0.01 pg Stx2a ml⁻¹ in milk has recently been introduced using mAb pair Stx2-1 and Stx2-2 (He et al., 2013). Such an assay can be a useful method for the routine diagnosis of Stx2 contamination in the milk-production process, thus reducing the risk of STEC outbreaks.

Over the last two decades, the resurgence of tuberculosis has been documented throughout the world, and much of this increase has coincided with HIV epidemics. An early diagnosis of tuberculosis is needed to initiate an early anti-tubercular therapy to avoid unnecessary morbidity and mortality (Mehta et al., 2012a). Recently, we developed an indirect sandwich I-PCR assay for the detection of Mycobacterium tuberculosis specific region-of-difference (RD) antigens: early secretory antigenic target-6 (ESAT-6; Rv number Rv3875), culture filtrate protein-10 (CFP-10; Rv3874), mycobacterial protein from species tuberculosis (MPT-64; Rv1980c) and culture filtrate protein-21 (CFP-21; Rv1984c) in biological specimens of pulmonary as well as extrapolumary tuberculosis patients (Mehta et al., 2012b). We could detect up to 0.1 fg purified M. tuberculosis RD antigens by I-PCR, which was 10⁻⁶ times more sensitive than an analogous ELISA.

Xylella fastidiosa, a bacterial plant pathogen, is known to cause significant losses in many economically important crops. Peroni et al. (2008) compared ELISA, PCR and immuno-capture PCR assays, as well as I-PCR, to detect X. fastidiosa for an accurate diagnosis of Citrus variegated chlorosis. As expected, I-PCR revealed the maximum sensitivity among the four assays examined with an LOD of only 10 bacterial cells, whilst the LOD with the other assays was 10⁶ bacterial cells.

Palaeomicrobiology allows the identification of causative agents of ancient diseases, their geographical distribution and study of the genetic evolution of micro-organisms (Tran et al., 2011). Molecular biology techniques are commonly used to detect micro-organism DNA, but the risk of contamination, chemical modification/fragmentation of DNA and presence of PCR inhibitors in ancient samples limit the ability to detect ancient infections. However, good conservation of proteins has been described recently with the classification of mammalian species using the dental pulp of modern and ancient individuals, which led the researchers to explore alternative methods based on antigenic protein detection. Malou et al. (2012b) devised an I-PCR method for the detection of Yersinia pestis protein antigens in ancient dental pulp specimens collected from Black Death victims. This method could detect protein concentrations 70 times lower than ELISA. Moreover, I-PCR was the most sensitive method, followed by PCR and ELISA, and a combination of these methods increased the overall capacity to identify Y. pestis in ancient dental pulp specimens.

Detection of parasitic antigens
Angiostrongylus cantonensis causes angiostrongyliasis, the most common cause of eosinophilic meningitis and meningoencephalitis in South-east Asia and the Pacific Basin (Baheti et al., 2008). A reliable diagnostic measurement of infection with A. cantonensis is difficult, because the parasitic nematode is unidentified in the cerebrospinal fluid of most of the afflicted patients and the sensitivity of ELISA for circulating worm antigens in patient sera is quite low (Chye et al., 1997). Chye et al. (2004) exploited a sandwich I-PCR assay in which both capture antibody and the detection antibody were mAbs for the detection of circulating 204 kDa AcL5 (fifth-stage larvae) antigens of A. cantonensis in the serum of patients with eosinophilic meningitis/meningoencephalitis with 100 % specificity. In contrast to ELISA, the sensitivity of I-PCR was also 100 % for patients known to have the parasitic worm in the cerebrospinal fluid and 97 % for patients who displayed only clinical syndromes. A single 150 bp DNA band was routinely observed with 0.1 ng purified antigen 1⁻¹ with I-PCR, whilst ELISA could not detect antigen concentrations >10 ng 1⁻¹, thus demonstrating that I-PCR was 10⁻⁶–10⁻⁵ times more sensitive than ELISA.

Detection of mycotoxins
Aflatoxin B₁ is a natural mycotoxin that enters the food chain by contamination of food grains and feedstuffs, potentially posing carcinogenic risks to animal and human health. I-PCR has the potential to direct the need of meeting the regulatory limits by detecting trace levels of aflatoxin present in food and animal feeds. Babu & Muriana (2011) described a real-time I-PCR assay for the quantification of aflatoxin B₁ suspended in a methanol:water solution that could also serve as an extraction solvent. I-PCR formats included direct sandwich versus indirect sandwich assays using mAbs versus polyclonal antibodies. Their assay could demonstrate the sensitive detection of aflatoxin B₁ from 10 p.p.b. down to 0.1 p.p.b. and represents a useful model system that can easily be adapted for aflatoxin detection in a variety of food or animal feed samples using a simple methanol:water solution as an extraction solvent.

Some of the various microbial antigens detected by I-PCR assays are summarized in Table 1.

Detection of antibodies to microbial antigens
Unlike microbial antigen detection, there are few reports available for the detection of circulating antibodies to microbial antigens by I-PCR assay. Mweene et al. (1996)
demonstrated an I-PCR assay for the detection of antibodies against bovine herpes virus type 1 in experimentally infected calf/rabbit serum samples with a 10^5-fold higher sensitivity than ELISA.

The detection of mumps-specific IgG demonstrates an important role in immunity surveillance, evaluating the efficacy of vaccination programmes, identifying susceptible cohorts in the population and designing future vaccination strategies. However, this poses a problem when measuring mumps-specific IgG prevalence in populations in which mumps virus infection is controlled by MMR (measles, mumps and rubella) vaccination. A quantitative real-time I-PCR assay was developed by McKie et al. (2002a, b) for the detection of mumps-specific IgG antibodies. The antibody responses to vaccination were lower than those following infection with WT virus, and the levels of antibody in the population were no longer boosted by continuing circulation of the virus. In contrast to results from other laboratories, the sensitivity and specificity of their real-time I-PCR assay did not exceed that of ELISA.

The most commonly used methods for the diagnosis of Q fever are serological tests, and the interpretation of these tests depends on the phenomenon of phase variation exhibited by Coxiella burnetii with a partial loss of LPS. The lag phase in antibody response of 7–15 days after clinical manifestations is the major drawback regarding serological diagnosis of acute Q fever. Recently, Malou et al. (2012a) compared the efficacy of I-PCR, PCR, ELISA and immunofluorescent antibody tests for the diagnosis of Q fever by the detection of phase II IgM anti-Coxiella burnetii. It was found that the highest sensitivity was achieved with I-PCR (87 %), followed by PCR (58 %), ELISA (38 %) and immunofluorescent antibody (32 %) tests.

Lyme disease caused by the slow-growing Borrelia burgdorferi is the fastest-growing zoonotic disease. Current assays for the detection of Borrelia burgdorferi infection are challenged by the standardization of antigen source and analysis subjectiveness. Therefore, Halpern et al. (2013) exploited an I-PCR assay using recombinant in vivo-expressed Borrelia burgdorferi antigens for the detection of a host immune response to Borrelia burgdorferi infection. The I-PCR format was a liquid-phase protein detection method using magnetic beads coated with intact spirochaetes, which provided an effective antigen presentation and demonstrated the detection of host-generated antibodies in experimentally infected mice at day 11 post-inoculation, whereas host-generated antibodies were detected at day 14 post-inoculation by ELISA and day 21 post-inoculation by Western blotting. Furthermore, magnetic beads coated with the recombinant Borrelia burgdorferi in vivo-expressed antigen outer surface protein C or Borrelia membrane protein A documented positive detection of host-generated antibodies in mice at day 7 post-inoculation with noticeably enhanced I-PCR signals above the background, thus suggesting that their assay had the potential for the sensitive detection of multiple host-response antibodies and isotypes to Borrelia burgdorferi infection.

We recently developed an indirect I-PCR assay for the detection of anti-ESAT-6, anti-CFP-10, anti-MPT-64 and anti-CFP-21 antibodies in the sera of pulmonary tuberculosis patients. However, paired sample analysis, that is, the detection of M. tuberculosis RD antigens and anti-RD antibodies together, in the biological samples of pulmonary tuberculosis patients by I-PCR assay exhibited better sensitivity and specificity compared with the detection of RD antigens or anti-RD antibodies (Mehta et al., 2012b).

As well as detecting microbial antigens and antibodies in various infectious diseases, I-PCR assays have also been exploited for the diagnosis of non-infectious diseases, for example, for the detection of phosphorylated Tau epitopes and amyloid peptide biomarkers from the neurons for early diagnosis of Alzheimer’s disease (Singer et al., 2009; Hashimoto et al., 2012).

Sensitivity and specificity are critical factors in I-PCR assays. Although I-PCR assays have been well documented for the ultraslow detection of target antigens in biological specimens, these assays are vulnerable to sense-non-specific signals and compromise over the accuracy of the test. This method remains unused as a routine clinical diagnostic test, as it does not meet the high criterion standards established by the Clinical and Laboratory Standards Institute requiring 99 % sensitivity and specificity (levels easily achieved by ELISA). The most serious problem preventing high sensitivity and specificity of I-PCR remains the high background noise due to non-specific binding and the sample matrix effect, which can be resolved to some extent by the exploitation of a liquid format using a nanoparticle-based assay.

Conclusion

This review has described the utility of the ultrasensitive I-PCR assay for the detection of potential microbial antigens, demonstrating its wide applications for early diagnosis of infectious diseases, especially for viral and prion infections, as well as for slow-growing and fastidious bacteria that are difficult to isolate. Although I-PCR assays are mostly restricted to target proteins, we can detect any non-nucleic acid molecule, such as lipids or carbohydrates. To further improve the I-PCR assay, a reduction in background noise in complex biological matrices, automation and a reduction in various steps, as well as the use of diminutive forms of protocols, is needed. There are several formats of I-PCR assays; the preferred is the sandwich real-time I-PCR, followed by magnetic bead/GNP-based real-time I-PCR. The use of magnetic beads/GNPs might provide a concrete solution to the reduction in background noise, the establishment of automated one-step I-PCR and hence a reduction of the overall duration of the assay. Although the cost of I-PCR assays is high, the applicability of these assays has been encouraged in recent years due to their ultra-sensitivity and robustness, which is required for the early detection of infectious as well as non-infectious diseases. However, further work is required to develop a reliable and
cost-effective I-PCR assay that can be used for routine use in resource-poor settings.

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References


