



Cite this: RSC Adv., 2017, 7, 51789

 Received 22nd August 2017  
 Accepted 25th October 2017

 DOI: 10.1039/c7ra09064a  
[rsc.li/rsc-advances](http://rsc.li/rsc-advances)

## Detection methods for *Pseudomonas aeruginosa*: history and future perspective

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*Pseudomonas aeruginosa* is a prevalent, opportunistic, Gram-negative bacterium that infects immunocompromised individuals, frequently causing hospital-acquired and community-acquired infections. Currently, *Pseudomonas aeruginosa* is one of the most widespread and fatal agents among the various causes of nosocomial infections. *P. aeruginosa* has been associated with increased mortality relative to *Staphylococcus aureus* or other Gram-negative in bloodstream infections. As few as 10–100 bacilli are capable of colonizing the intestine of critically ill or immunocompromised patients, therefore, early detection of *Pseudomonas aeruginosa* is particularly important. Here, we have summarized and analyzed the development of detection techniques for *Pseudomonas aeruginosa* over the past 50 years. We also discuss the prospects for future research on *Pseudomonas aeruginosa* detection methods in the hope of providing a reference for relevant studies.

### 1. Introduction

*Pseudomonas aeruginosa* is a widespread, opportunistic, Gram-negative bacillus that causes various clinical infections and severe infectious diseases.<sup>1–3</sup> Currently, it is one of the most troublesome multidrug-resistant bacterial causes of nosocomial infections; infection with *Pseudomonas aeruginosa* can cause

long-term chronic diseases, particularly affecting immunocompromised (especially neutropenic) patients or those admitted to the intensive care unit (ICU).<sup>4–6</sup> In food testing, the WHO has identified *Pseudomonas aeruginosa* as an indicator of drinking water quality while the European Communities and the Codex Alimentarius Commission also stipulated that *Pseudomonas aeruginosa* should not be detected in water.<sup>7,8</sup> In addition, *Pseudomonas aeruginosa* can cause infections at very low concentrations. Hence, early detection is critical for treating *Pseudomonas aeruginosa* infection. Scientists have long been committed to establishing a rapid and sensitive detection method for *Pseudomonas aeruginosa*. Currently, numerous modern detection approaches have been developed, such as flow cytometry (Rüger *et al.*, 2014),<sup>9</sup> immunological detection

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methods<sup>10</sup> and molecular biology-based detection methods.<sup>11</sup> However, conventional culture methods are still the most commonly applied methods in clinical practice at present. Therefore, these new detection approaches still need to be further improved to achieve large-scale applications in clinical practice. Here, we review the development, advantages, and disadvantages of *Pseudomonas aeruginosa* detection methods.



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## 2. Conventional bacterial culture methods

Conventional *Pseudomonas aeruginosa* detection methods are based on the biological characteristics of the bacterium under certain culture conditions, such as Gram-negative or Gram-positive status, or the activities of bacterial molecules such as oxidase, acetamidase, arginine dihydrolase, and pyocyanin. In 1955, Lowbury *et al.* developed a selective medium for *Pseudomonas aeruginosa* that is supplemented with 0.03% cetrimide. The medium has very high selectivity and subsequently became one of the most common selective media for *Pseudomonas aeruginosa*.<sup>12</sup> In 1965, Brown *et al.*<sup>13</sup> developed an improved selective medium containing cetrimide, based on previous studies, and demonstrated that the Lemco-based selective medium (CTA1) promoted better pyocyanin production. Growth in selective medium with King's medium B as the base (CTA2) resulted in stronger fluorescence at an incubation temperature of 42 °C, while other (non-*Pseudomonas aeruginosa*) species did



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not produce fluorescence at this temperature. Therefore, the CTA2 medium can serve as a diagnostic medium specific for *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* can produce ammonia as a nutrient by breaking down acetamide, an ability that is absent in other bacteria. Based on this mechanism, Szita *et al.*<sup>14</sup> (1990) developed a liquid synthetic medium in 1990 that enables higher detection selectivity, sensitivity, and speed than culture medium supplemented with cetrimide. In addition, many other researchers have investigated the feasibility of using cetrimide as an additive to identify *Pseudomonas aeruginosa*.<sup>15-17</sup> Infection with *Pseudomonas aeruginosa* can cause long-term chronic diseases. Long diagnostic turnaround time can reduce patient outcomes and increase hospital costs. The automated systems promise shorter turnaround times to diagnostic results and are widely used in many clinical laboratories for identification of bacterial species and antimicrobial susceptibility testing (AST). The automated systems have many advantages, such as high degree of automation with a simple operating procedure, improved specimen handling, good reproducibility and accuracy, *etc.* Vitek 2 (BioMérieux, France),<sup>18</sup> Phoenix 100 (BD Biosciences, USA)<sup>19</sup> and MicroScan WalkAway (Dade Behring, Inc., USA)<sup>20</sup> are the common automated identification systems currently used in China. The Vitek 2 is one of the earliest and most commonly used automated identification systems.<sup>21</sup> These automated systems not only identify *Pseudomonas aeruginosa* but are also capable of performing AST. Many scientists have used these instruments to analyze different sources of *Pseudomonas aeruginosa* and tested their identification and misidentification by comparing them with conventional methods (Table 1). For example, Bruins and his colleagues compared direct inoculation by Vitek 2 and the standard method; a total of 33 isolates of *Pseudomonas aeruginosa* were tested, and 78.8% were correctly identified (Bruins *et al.*, 2004).<sup>22</sup> Saiman's result showed that only 57% (108 of 189) of nonmucoid strains and 40% (24 of 60) of mucoid strains were definitively identified as *Pseudomonas aeruginosa* with MicroScan Autoscan.<sup>20</sup> In 2003, a high rate of agreement between the Phoenix and the conventional methods was observed for *Pseudomonas aeruginosa* identification, and the rate of agreement

was 90.9%.<sup>23</sup> The same system showed different results in different studies, which might be due to different sample sources or experimental conditions.<sup>25-28</sup> Although automated identification systems have been clinically used to identify a variety of microbial species, these systems have a low rate of accuracy in the identification of *Pseudomonas aeruginosa*.<sup>18,20,24</sup> Clinical laboratories should be aware of the problem with the automated systems in testing *Pseudomonas aeruginosa* and seek alternatives, validated methods for routine use.

### 3. Immunological assays

Immunological techniques utilize the highly specific binding between antibodies and antigens and facilitate qualitative or quantitative detection based on specific reactions resulted from antigen–antibody binding. Modern immunoassay techniques have achieved high-sensitivity detection and have been developed into multiple types of immunoassay methods by introducing enzyme-catalyzed reactions, fluorescence, or isotope labeling as a specific measure of antigen–antibody binding.<sup>29,30</sup> While reviewing the literature, we found that the main immunoassays currently applied for detecting *Pseudomonas aeruginosa* include enzyme-linked immunosorbent assays (ELISAs),<sup>31</sup> immunoblotting (IBT),<sup>32</sup> immunofluorescence,<sup>33</sup> immunoelectrophoresis<sup>34</sup> and solid-phase radioimmunoassay,<sup>35</sup> which are discussed briefly below.

#### 3.1 Enzyme-linked immunosorbent assays (ELISAs)

ELISAs are based on an immunological technique that utilizes enzyme-catalyzed reactions to enhance the sensitivity of the specific antigen–antibody reaction.<sup>30</sup> The underlying principle is that antigens or antibodies with immunological activity are immobilized on the surface of a solid-phase carrier, and then the antigen or antibody sample to be detected is reacted with the immobilized antigen or antibody molecules, followed by the addition of enzyme-labeled antigen or antibody molecules prior to incubation. Finally, enzyme-catalyzed substrates are added for luminescence detection<sup>31</sup> and the enzymatic reaction plays

Table 1 Results of direct inoculation using Vitek 2, Phoenix 100 and MicroScan WalkAway compared with the conventional method<sup>a</sup>

Vitek-2		Phoenix 100		MicroScan WalkAway		References
Correctly/tested identified	Correct identification rate (%)	Correctly/tested identified	Correct identification rate (%)	Correctly/tested identified	Correct identification rate (%)	
26/33	78.8					22
21/21	100					21
98/105	93.3					26
88/146	60.3					18
8/9	88.9					24
		12/12	100	6/9	66.7	24
		20/22	90.9	132/249	53.0	20
		5/7	71.4	8/10	80.0	25
		55/55	100			19
						23
						27
						28

<sup>a</sup> The correct identification don't contained low discrimination.



a role in signal amplification. Currently, ELISAs has been widely applied in the detection of pathogens and food-borne bacteria. It is also considered as one of the most successful detection techniques over the past few decades.

ELISAs were first used by Ueda *et al.* to detect immunoglobulin M (IgM) and immunoglobulin G (IgG) in horse serum against common serological *Pseudomonas aeruginosa* antigens (protease and elastase).<sup>36</sup> With their method, horseradish peroxidase (HRP)-labeled rabbit anti-horse IgM and IgG were used as conjugated enzyme-labeled antibodies, while 5-aminosalicylic acid and H<sub>2</sub>O<sub>2</sub> were used as HRP substrates for signal amplification, thereby enabling the detection of IgM and IgG in colt and racehorses. Granstrom *et al.* developed a specific ELISA-based detection method against antibodies from patients infected with *Pseudomonas aeruginosa*, using 4 extracellular proteins as antigens: exotoxin A, elastase, alkaline protease, and phospholipase C.<sup>37</sup> The method was tested on samples from 39 burn patients, and the results showed that the detection of antibodies against exotoxin A and phospholipase C by ELISA could be used to monitor *Pseudomonas aeruginosa* infection in patients. Fomsgaard *et al.*<sup>38</sup> reported the use of anti-lipopolysaccharide (LPS) antibodies to develop an ELISA method for the quantitative detection of *Pseudomonas aeruginosa* in cystic fibrosis (CF) patients with chronic infection. The results of their study showed that expression levels of anti-LPS antibodies (IgG and IgA) significantly increased during the early stage of *Pseudomonas aeruginosa* lung infection, and they continued to increase to very high levels during later stages of infection. IgM levels increased during early infection but did not continue to increase in the later stages of infection. Fomsgaard *et al.* also assessed the diagnostic efficiency of this method for detecting early chronic infection with *Pseudomonas aeruginosa* by comparison with the immunoelectrophoresis method. Their results showed that the method targeting IgG and IgA antibodies had 86% and 89% positive predictive values, respectively, and has 98% and 97% negative predictive values, respectively. The detection rate using IgM as the target was low. Dogru *et al.* (2013) assessed the utility of ELISA in detecting early *Pseudomonas aeruginosa* infection in CF patients through comparison with culture methods. The study involved 90 CF patients with long-term follow-up.<sup>39</sup> The results showed that the ELISA method had a higher sensitivity in detecting anti-*Pseudomonas aeruginosa* antibodies, and thus, these antibodies can be used as markers for early diagnosis. Antibodies against alkaline protease showed the highest specificity, while anti-elastase antibodies showed the highest sensitivity. Although specificity is one of the major advantages of ELISA, many secreted *Pseudomonas aeruginosa* proteins share high homology with those of other bacteria, raising the possibility of false-positive detection. For example, Beutin *et al.* (1996) reported that toxins from *Pseudomonas aeruginosa* could induce false-positive results when assaying for Shiga-like cytotoxins, using a commercial ELISA kit.<sup>40</sup>

ELISA methods have not only been applied for PA detection but also been used for the diagnosis of other lethal infections as well. For example, Cyrille *et al.* (2017)<sup>41</sup> reported the detection of HIV, HBV and HCV with 93.39%, 98.90% and 91.86%

sensitivity, respectively. In another study, Bouba *et al.* (2017)<sup>42</sup> successfully detected Zika virus (ZIKV) in 1084 blood donors from Cameroon by using the EuroImmun anti-NS1 IgG ELISA detection kit. Thus, despite the specificity issue, ELISA is an important technique which has been widely used for the detection of pathogens.

### 3.2 Enzyme-linked immunoelectrotransfer blots (IBTs)

IBTs, also known as enzyme-linked immunoelectrotransfer blots, are immunoassays involving the integration of gel electrophoresis and immunohistochemistry.<sup>43</sup> IBTs involve the separation of protein samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to a nitrocellulose membrane as the solid-phase carrier, using electric transfer system.<sup>44</sup> Subsequently, specific antibodies and enzyme-labeled antibodies are added successively, and finally, an enzyme-catalyzed substrate is added to produce a colored or luminescent product for detection purposes.<sup>45</sup> This method integrates the high resolution of SDS-PAGE and high specificity of immunoassays; therefore, it is very suitable for the highly sensitive detection of target proteins among different complexes (Hamid *et al.*, 2009; Párishamelin *et al.*, 1999; Chin *et al.*, 1991; Ayala-Sulca *et al.*, 2015).<sup>43-46</sup> The chronic infection caused by *Pseudomonas aeruginosa* can be fatal for CF patients. Shand *et al.* (1988) prepared a standard antigen (StAg) of *Pseudomonas aeruginosa* to serve as a basis for developing the IBT method with serum antibodies. A comparison with the crossed immunoelectrophoresis (CIE) method showed that the sensitivity of the IBT method was approximately 2 orders of magnitude higher than that of the CIE method, with good reproducibility. The results of their study also showed that IBT could be developed into a highly sensitive assay for detecting early *Pseudomonas aeruginosa* infection in CF patients. Caballero<sup>47</sup> reported an improved IBT method on the basis of Shand's study,<sup>32</sup> which was successfully tested with anti-*Pseudomonas aeruginosa* antibodies in patients with bronchiectasis. Weisner *et al.*<sup>48</sup> utilized the IBT technique to develop a method that could detect the A-band LPS antibody of *Pseudomonas aeruginosa* in oral fluid samples.

Besides many applications of IBTs in *Pseudomonas aeruginosa* infection, recent past also illustrates the pace at which the IBTs based detection of other diseases obtained valuable implementations and is advancing. For example, Eduardo *et al.* (2015) detected human cysticercosis which utilized purified native antigen mix from cysticercus fluid of *Taenia solium* by using ITBs techniques, and results showed that the purified native antigen mix improved the diagnostic efficiency of the IBTs test.<sup>46</sup>

### 3.3 Immunofluorescence methods

Although immunoenzyme techniques have good sensitivity, their relatively tedious procedures have limited further development. However, immunofluorescence methods developed on the basis of immunological techniques involve simpler operating procedures with a good sensitivity and specificity, thus, they are considered to be among the most



promising assays for pathogens (Pemberton *et al.*, 2014; Miyazaki *et al.*, 2014; Parks *et al.*, 1983).<sup>49–51</sup> In addition, the development of nanotechnology has brought new opportunities for fluorescence assays. For instance, nanomaterials developed in recent years, such as fluorescence quantum dots,<sup>52</sup> nanoclusters,<sup>53</sup> fluorescent rare-earth complexes,<sup>54</sup> and internalizable fluorescent materials,<sup>55</sup> offer the advantages of high fluorescence efficiency, durable fluorescence, and tunable fluorescence emission wavelengths.<sup>56</sup> Shibuya *et al.* (1987) used the immunofluorescence technique for the first time to perform rapid *in situ* detection of *Pseudomonas aeruginosa* in sputum samples.<sup>33</sup> Pfaller *et al.*<sup>57</sup> developed and evaluated a 1-step immunofluorescence method for the direct detection of *Pseudomonas aeruginosa* in a blood culture sample and used this method to perform statistical analysis on the sample. The method enabled effective and rapid detection of *Pseudomonas aeruginosa*. Tang *et al.* (1992)<sup>58</sup> established a direct immunofluorescent antibody-staining method for detecting *Pseudomonas aeruginosa* in sputum samples using serum-specific monoclonal antibodies, in order to assess the status of pulmonary infection. The results showed that this method had a detection limit of 10 colony-forming units (CFU) per mL and could be completed within 3 h. However, the greatest drawback of immunofluorescence methods is a low signal-to-noise ratio, which may lower its detection specificity. Immunofluorescence technique is steadily improving with the passage of time. Recently, David *et al.* (2014) demonstrated that automated-multiplex immunofluorescence immunoassay (InoDiag) test owned similar sensitivities (65.5%) between immunofluorescence, but better specificities (95.1–98%) than enzyme-linked immunosorbent assays (ELISAs), through detecting specific anti-C. trachomatis immunoglobulin G.<sup>59</sup> Furthermore, Helle *et al.* (2016) verified that immunofluorescence assay was significantly more sensitive than microscopy of iodine-stained concentrates using either formol-ethylacetate assay or salt-sugar flotation.<sup>60</sup>

The developed immunoassays for *Pseudomonas aeruginosa* also include convective immunoelectrophoresis, which was developed by Wagner *et al.*<sup>34</sup> and solid-phase radioimmunoassay, established by Kohler *et al.*<sup>35</sup> These immunoassays have provided abundant approaches for the highly sensitive detection of *Pseudomonas aeruginosa*.

## 4. Molecular biology assays

Alongside the development of polymerase chain reaction (PCR) in the 1980s and the continuous progression of molecular biology methods,<sup>61</sup> scientists have established numerous effective molecular biology assays that have been widely applied for detecting pathogens and have provided a technical basis for epidemiological investigations,<sup>62</sup> pathogen-specific detection,<sup>63</sup> and pathogen genotyping.<sup>64</sup> With the development of molecular biology techniques, molecular biology-based methods for rapidly detecting *Pseudomonas aeruginosa* have also developed rapidly.<sup>65,66</sup> Here, we summarize the development of molecular biology-based *Pseudomonas aeruginosa* assays.

### 4.1 Conventional PCR methods

The PCR technique is one of the most important inventions over the past 3 decades. In recent years, PCR-based assays for *Pseudomonas aeruginosa* also have developed progressively. Kingsford *et al.*<sup>65</sup> established a PCR detection method for *Pseudomonas aeruginosa* that specifically targets the 16S rRNA gene. The method allows the detection of 1 pg chromosomal DNA or  $1 \times 10^5$  CFU mL<sup>-1</sup> of *Pseudomonas aeruginosa*. Detection sensitivity can be increased up to 1 fg chromosomal DNA or 10 CFU mL<sup>-1</sup> if detection is based on the use of a fluorescent probe. Hummel and Unger<sup>66</sup> established the first PCR method that specifically detected *Pseudomonas aeruginosa* based on the exotoxin A gene and evaluated the effectiveness of this method for rapid *Pseudomonas aeruginosa* detection in mechanically ventilated patients. The results showed that the PCR method targeting exotoxin A gene detected 57 positive samples out of 364 total samples, whereas the conventional culture method only detected 36 positive samples, indicating that the exotoxin A gene-based PCR method had higher sensitivity. Alongside the continuous development of PCR assays for *Pseudomonas aeruginosa*, an increasing number of specific genes have been discovered, such as *ecfX*, *gyrB*, *algD* GDP mannose, *oprL*, and *fliC*.<sup>67</sup> Specificity is critical for the success of conventional PCR, but is also the most important cause of failure in PCR detection. Therefore, many researchers have investigated the specificity of different *Pseudomonas aeruginosa* genes. For example, Dev Vos *et al.* (1997) examined the specificity of the *oprL* gene in *Pseudomonas aeruginosa* detection.<sup>68</sup> Further information on the specificity of tests for *Pseudomonas aeruginosa* genes is presented in Table 2. It can be seen from Table 2 that except for *fliC* gene, which shows low

Table 2 Statistics of a specificity test of target genes for detecting *P. aeruginosa*<sup>a</sup>

PCR target gene	Accuracy of <i>P. aeruginosa</i> -detection assay			
	Positive samples	Negative samples	Year	Ref.
16s rDNA	59/59	1/15	2007	69
	63/63	2/28	2009	73
<i>algD</i> GDP mannose	101/103	0/87	2003	71
	176/176	0/26	1999	72
<i>ETA</i>	62/63	0/28	2009	73
	108/113	0/87	2003	71
<i>ecfX</i>	63/63	0/28	2009	73
	59/59	0/15	2007	69
<i>fliC</i>	59/59	13/15	2007	69
<i>gyrB</i>	59/59	0/15	2007	69
	63/63	0/28	2009	73
	113/113	0/87	2003	71
<i>oprL</i>	40/40	0/18	2001	75
	63/63	4/28	2009	73
	59/59	2/15	2007	69
	128/138		2016	76
<i>toxA</i>	55/59	0/15	2007	69
<i>oprI</i>	128/138		2016	76

<sup>a</sup> Statistical analysis was performed according to Deschaght *et al.* (2011).



specificity, all of the other genes have very high specificity, especially the *ecfX* and *gyrB* genes, whose detection was not associated with false positive or false negative results.<sup>70,73</sup>

Conventional PCR was utilized widely for its developed procedure to obtain consequence of reliability and stability. Recently, PCR-based assays for clinical diagnosis and ensuring food safety have been developed progressively. For instance, Bobbi *et al.* (2016) detected routine clinical diagnostic specimens from patients in the USA with PCR through targeting the *oppA1* gene of *B. burgdorferi* sensu lato and identified positive specimens.<sup>77</sup> The combined use of conventional PCR, magnetic particles and chemiluminescence technology for the development of efficient biosensors for clinical purpose is an emerging trend. Highly sensitive and rapid detection of *Pseudomonas aeruginosa* was achieved using conventional PCR, magnetic particles (MNPs), and chemiluminescence technology.<sup>78,79</sup> The

results showed that *Pseudomonas aeruginosa* was successfully detected with detection limit as low as 7.5 fM in term of *gyrB* fragments. Furthermore, Tang *et al.* (2014) devised a nested PCR method based on MNPs to detect *Pseudomonas aeruginosa*.<sup>80</sup>

#### 4.2 Real-time, fluorescence-based quantitative PCR

In 1996, Applied Biosystems, Inc. (USA) invented the real-time, fluorescence-based quantitative PCR (real-time qPCR) method that enables quantitative detection *via* real-time monitoring of PCR reactions by introducing fluorescent molecules into the reaction mixture. Currently, it has become one of the most widely used nucleic acid-based molecular detection techniques for pathogens.<sup>81</sup> Qin *et al.*<sup>71</sup> established a multiplex real-time PCR assay that simultaneously and specifically detected the 16S rRNA and *gyrB* genes of *Pseudomonas aeruginosa* in CF patients, and validated their reliability and sensitivity as compared with other specific genes, such as *oprI*, *exoA*, and *algD*. The method enabled detection of *Pseudomonas aeruginosa* in CF patients within a shorter period. Motoshima *et al.*<sup>74</sup> developed a real-time PCR method using melting curve analysis (MCA), which specifically targeted the *gyrB* gene. As shown in Fig. 1, this method could readily distinguish *Pseudomonas aeruginosa* from other bacteria. Comparison between the performance of the real-time PCR method and the Vitek detection system, using 224 Gram-negative bacterial samples, confirmed that this method could accurately distinguish *Pseudomonas aeruginosa* from other Gram-negative bacteria within 3 h. A duplex real-time PCR assay was established against 2 specific genes, *ecfX* and *gyrB*, to effectively address the false-positive and false-negative issues in detecting *Pseudomonas aeruginosa*.<sup>60,73</sup> Le Gall *et al.* (2013)<sup>82</sup> demonstrated that multiplex real-time PCR could effectively solve the contradiction between sensitivity and specificity by analyzing the specificity and sensitivity of different genes, in addition to enabling detection of *Pseudomonas aeruginosa* from a mixed bacterial sample. Real-Time fluorescence-based PCR was also a highly sensitive, powerfully rapid, widely useful and prospectively detected tool in respiratory tract infections and pneumonia leading pathogen. Naoyuki *et al.* (2015) tested diagnostic sensitivity of immunochromatographic assay which was a rapid antigen kit for detection of *Mycoplasma pneumonia*, and compared with real-time PCR as gold standard.<sup>83</sup> Idrissa *et al.* (2016) described a duplex real-time PCR assay based on SYBR dye for detection of *Neisseria meningitidis* and *Streptococcus pneumonia* in the cerebrospinal fluid.<sup>84</sup> Although most *Pseudomonas aeruginosa* tests have been performed with samples from CF patients, numerous researchers use real-time PCR to detect *Pseudomonas aeruginosa* in other samples. For example, Schwartz *et al.* have detected *Pseudomonas aeruginosa* in environmental wastewater,<sup>85</sup> Feizabadi *et al.* tested saliva and bronchial secretions,<sup>86</sup> and Lee *et al.* established a *Pseudomonas aeruginosa*-detection method for chlorinated water and aerosols.<sup>87</sup>

#### 4.3 Multiplex PCR

The main advantage of multiplex PCR is its ability to simultaneously amplify multiple PCR products in a single reaction,

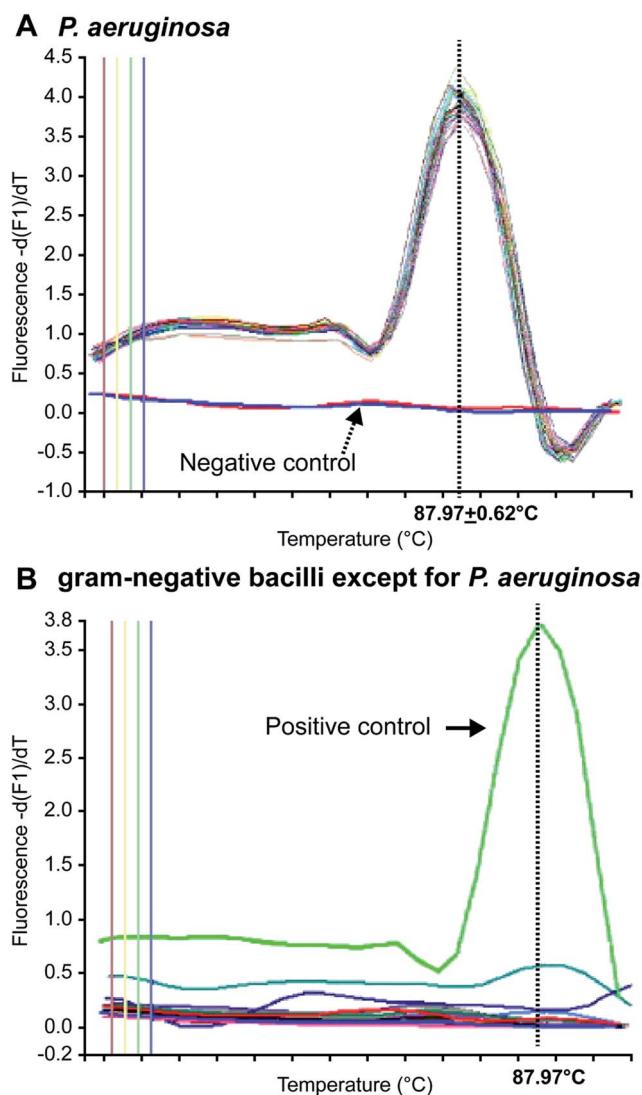


Fig. 1 MCA of Gram-negative bacilli. (A) *Pseudomonas aeruginosa*-positive samples provided sharp peaks with a constant  $T_m$  of  $87.9^\circ\text{C}$ , and (B) non-*Pseudomonas aeruginosa* samples showed no peak at  $87.9^\circ\text{C}$ . (adapted from Motoshima *et al.*, 2007). Order number from the Copyright Clearance Center: 4066350162315.



thereby enabling multiplex detection and significantly reducing the detection cost and time requirements.<sup>88</sup> Microorganism-induced diseases are usually complicated with infections by multiple pathogens, for instance, bacteria can easily enrich and grow on the walls of catheters in patients with mechanical ventilation and renal intubation, leading to mixed infections with multiple bacteria, but a singleplex PCR assay cannot be used to simultaneously detect multiple bacterial species.<sup>89,90</sup> Several studies have been conducted to establish multiplex PCR assays that include *Pseudomonas aeruginosa*. De Vos *et al.*<sup>68</sup> simultaneously detected *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* in clinical samples using a duplex PCR assay. This method had 100% sensitivity and 74% specificity, with a detection limit of  $10^2$  cells per mL in skin biopsy specimens from patients with burns and sputum samples from CF patients. Da Silva Filho *et al.* developed a multiplex PCR assay that simultaneously detected *Pseudomonas aeruginosa*, *Burkholderia cepacia*, and *Stenotrophomonas maltophilia* in the saliva and respiratory samples from CF patients (Da *et al.*, 2004).<sup>91</sup> British scholars, Fothergill *et al.* (2008)<sup>92</sup> reported development of a multiplex PCR method that differentiated Liverpool epidemic strain (LES), Midlands1 (Mid1) strain, and Manchester epidemic strain (MES) with 100% specificity and sensitivity. Thong *et al.* developed a multiplex PCR assay that simultaneously detected methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*.<sup>93</sup> They also optimized the PCR conditions to ensure the specificity of amplification and confirmed that this method had 100% accuracy for both positive and negative samples by testing it with 50 culture samples. To better guarantee the detection specificity, Salman *et al.* (2013) developed a multiplex PCR assay for simultaneous amplification of 4 specific genes: *gyrB*, 16s rRNA, *oprL*, and *ETA*.<sup>94</sup> Aghamollaei *et al.* (2015) developed a highly specific and sensitive method to detect *Pseudomonas aeruginosa* based on triplex PCR that amplifies the *lasI*, *lasR*, and *gyrB* genes.<sup>95</sup> These methods could improve the specificity, but are inconvenient and have a relatively high cost in clinical applications. In 2017, Jiang *et al.* developed a combination of multiplex polymerase chain reaction (PCR) and capillary electrophoresis (MPCE) assay to detect thirteen bacterial pathogens responsible for lower respiratory tract infections. The result showed that the specificity and sensitivity for *Pseudomonas aeruginosa* were 100%.<sup>96</sup> Multiplex PCR was a widely useful tool for detection of respiratory pathogens and transmitted infection in clinical specimens as well. Tayoun *et al.* (2015) described a highly sensitive, rapid and affordable sample-to-answer multiplex PCR assay for simultaneous detection of *Trichomonas vaginalis*, *Nesseria gonorrhoeae*, and *Chamydia trachomatis* resulting in sexually transmitted infections (STIs).<sup>97</sup>

#### 4.4 Isothermal amplification techniques

Isothermal amplification techniques have overcome the shortcomings of conventional PCR methods, which require thermocycling for amplification. Isothermal amplification enables the rapid amplification of nucleic acid molecules at a constant

temperature, which has not only reduced the equipment demand, but also has a higher sensitivity.<sup>98</sup> Some recent studies have reported use of the loop-mediated isothermal amplification (LAMP) technique to detect *Pseudomonas aeruginosa*. Goto *et al.* developed a LAMP assay based on a hydroxynaphthol blue (HNB) colorimetric assay (Fig. 2), which targeted the *oprL* gene of *Pseudomonas aeruginosa* specifically. The results showed that the LAMP assay had 100% specificity for the serogroup with a  $>10$ -fold greater sensitivity than conventional PCR. It could detect 130 CFU per 0.1 g of mouse feces or 3.25 CFU per reaction. In addition, performing the assay only required 2 h from DNA extraction to detection.<sup>99</sup> Moreover, this technique was also used to detect other organisms. For example, Liu *et al.* (2017) described a sensitive, reliable LAMP method to detect *Neisseria gonorrhoeae* *porA* pseudogene, and their results showed that the sensitivity and specificity of the LAMP assay were 94.7 and 85.7%, related to traditional culture, respectively.<sup>100</sup>

#### 4.5 Fluorescence *in situ* hybridization (FISH) technique using peptide nucleic acid (PNA) probes

FISH based on PNA probes is a rapid diagnostic technique for pathogens. PNA probes are more conducive to hybridization than oligonucleotides owing to their neutral charges. The targets of PNA probes are the rRNA molecules, which are abundant in nature, and thus do not need to be amplified during the detection of single microorganisms (Parcell *et al.*,

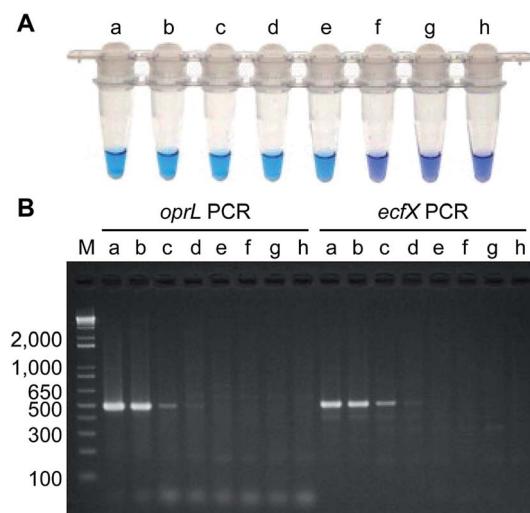


Fig. 2 Comparison of the sensitivities of LAMP and PCR assays using *Pseudomonas aeruginosa*-inoculated fecal samples. (A) Sensitivity of LAMP for *Pseudomonas aeruginosa* as determined by measuring HNB color changes. A positive reaction was indicated by the change in color from violet to sky blue. (B) Agarose gel electrophoretic analysis of PCR products obtained after 35 cycles. The expected sizes of the PCR products obtained by PCR-amplification of the *oprL* and *ecfX* genes were 504 bp and 528 bp, respectively. Lanes (tubes) (a)  $1.3 \times 10^6$ ; (b)  $1.3 \times 10^5$ ; (c)  $1.3 \times 10^4$ ; (d)  $1.3 \times 10^3$ ; (e) 130; (f) 13; (g) 1.3 CFU; h, no *Pseudomonas aeruginosa*; M, DNA size marker. (adapted from Goto *et al.*, 2010). Order number from the Copyright Clearance Center: 4066350607817.



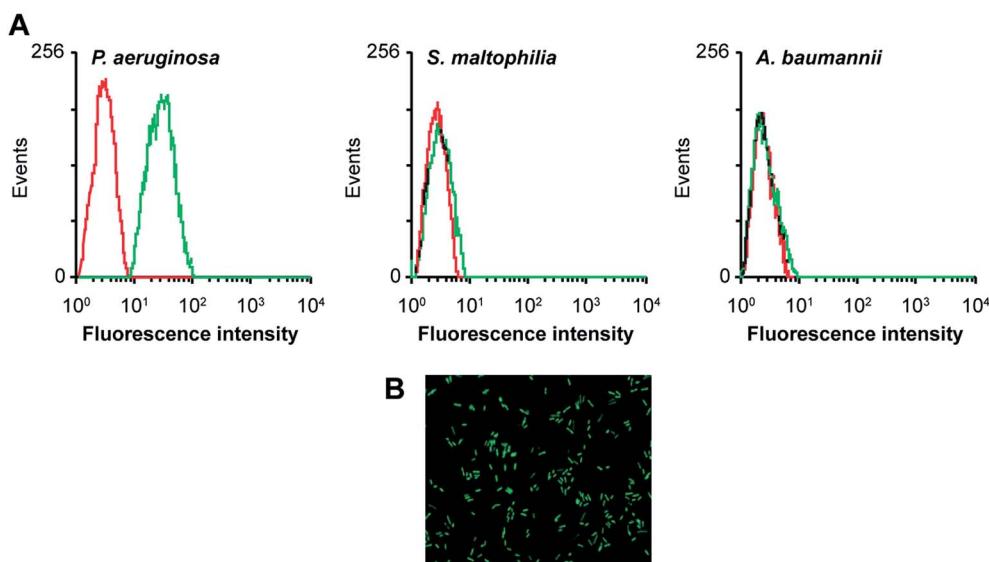


Fig. 3 Evaluation of the specificity of an aptamer probe. (A) Flow cytometry assay for the binding of the fluorescein isothiocyanate (FITC)-labeled F23 aptamer with *Pseudomonas aeruginosa* (target bacteria), *S. maltophilia* (negative bacteria) and *A. baumannii* (negative bacteria). The red curves represent the background binding of an unselected DNA library. The aptamer concentration in the binding buffer was 400 nM. (B) Evaluation of the specificity of the aptamer probe by FISH. Bacteria were hybridized with the FITC-labeled F23 aptamer probe. A representative image of cultured *Pseudomonas aeruginosa* stained with the aptamer probe is shown. Green fluorescence signifies positive hybridization. (adapted from Wang *et al.*, 2011). Order number from the Copyright Clearance Center: 4066351239887.

2013).<sup>101</sup> In addition, PNA probes are barely affected by impurities in clinical samples; therefore, they have higher efficiency in directly detecting pathogens in clinical samples, such as blood, saliva, and wound cultures (Almeida *et al.*, 2013).<sup>102</sup> Søgaard and his colleagues evaluated the feasibility of using FISH with PNA probes to detect *E. coli* and *Pseudomonas aeruginosa* in clinical blood cultures.<sup>103</sup> The results showed that the method had 94.1% sensitivity and 99% specificity in detecting *Pseudomonas aeruginosa*. Peleg *et al.* developed a multiplex PNA FISH technique for the simultaneous detection of *Acinetobacter* and *Pseudomonas aeruginosa*, using dual-color fluorescence, which could detect the target bacteria in a mixture consisting of 10 different bacterial species.<sup>104</sup> The technique was tested with 60 clinical samples, and the results showed that its sensitivity and specificity for detecting *Pseudomonas aeruginosa* were 100% and 95%, respectively. Although this method is very simple, it still involves a culture period for enrichment to aid the detection of *Pseudomonas aeruginosa* during early infection.

#### 4.6 Aptamer-based *in situ* detection methods

Aptamers, also known as “artificial antibodies,” are short nucleic acid or peptide sequences capable of binding to a target molecule with high specificity and affinity (Ruff *et al.*, 2012).<sup>105</sup> Aptamers have several advantages over antibodies, such as easy production, easy storage, easy modification, and low cost. In addition, aptamers exhibit greater affinity and specificity for targets. Therefore, aptamers have been widely applied for detecting pathogens in recent years.<sup>106,107</sup> Wang *et al.* (2011) selected a bacteria-targeting ssDNA aptamer that specifically bound *Pseudomonas aeruginosa* and utilized its specific binding capacity to develop a rapid FISH method for detecting

*Pseudomonas aeruginosa* (Fig. 3). The results showed that the method had excellent specificity, and the entire detection process took 1.5–2 h. Hence, the method can potentially facilitate clinical *Pseudomonas aeruginosa* detection.<sup>108</sup>

#### 4.7 PCR-ELISA methods

Kurupati *et al.* used the PCR-ELISA DIG Labelling<sup>plus</sup> kit (Roche Diagnostics Ltd, Lewes, UK) to establish a method for detecting *Pseudomonas aeruginosa* in blood cultures by targeting the *oprI* gene.<sup>109</sup> The principle of the method is to first amplify the digoxin-labeled PCR product, which is then hybridized with a biotinylated probe. The resulting hybridization product is immobilized in a microwell plate *via* a biotin-streptavidin coupling system, followed by incubation with an anti-digoxigenin peroxidase conjugate. Finally, the substrate molecule, 2,2-azino-di-3-ethylbenzthiazoline sulfonate, is added to enable detection. Using this method, the authors correctly identified 73 positive samples and 42 negative samples with 100% specificity and sensitivity. The method could shorten the blood culture time from 2–3 days to 6–8 h. Furthermore, other pathogens were also detected using this technique. For example, Medeiros *et al.* (2017) reported that kDNA PCR-ELISA exhibited satisfactory precision for detection of *Leishmania infantum* in 14 peripheral blood samples from immunocompetent patients.<sup>110</sup>

### 5. Electrochemical assays

Electrochemical analysis is a rapid and sensitive analytical method involving a compact device that enables qualitative and quantitative detection, using the electrochemical properties of materials.<sup>111</sup> Liu *et al.* (2011) designed an electrochemical DNA



biosensor based on stem-loop-structured probes to detect *Pseudomonas aeruginosa*.<sup>112</sup> The principle of this method entails opening of the stem-loop after hybridization, so that the biotin molecule on the probe can bind streptavidin-labeled horse-radish peroxidase (SA-HRP) in solution, after which HRP catalyzes the production of an electrochemical signal. Sensitivity test results showed that the method could be used to detect 16S rRNA at a concentration as low as  $0.012 \text{ pg } \mu\text{L}^{-1}$ , with a linear relationship between  $0.3\text{--}600 \text{ pg } \mu\text{L}^{-1}$ . Webster *et al.* (2014) reported a disposable carbon electrode sensor that could directly detect pyocyanin production by *Pseudomonas aeruginosa* in samples.<sup>113</sup> The electrochemical detection of *Pseudomonas aeruginosa* pyocyanin in samples could be completed within 5 min, and the entire procedure does not require sample-preparation and -isolation steps. Furthermore, the method has exhibited excellent sensitivity and specificity (Fig. 4). In the same year, Sismait and colleagues improved the sensitivity of square wave voltammetry (SWV) in analyzing pyocyanin production by supplementing the culture with tyrosine and valine as positive regulators of pyocyanin.<sup>114</sup> Sensitive detection of quorum sensing can potentially enable early detection of *Pseudomonas aeruginosa* and facilitate early medical intervention. Sensitive detection of quorum sensing (QS) molecules has the potential for early identification of *Pseudomonas aeruginosa* and, thereby, facilitating early medical intervention. Shang *et al.* (2014) developed a 1-step method to prepare thiazole termed IQS, which is a recently isolated cell-cell communication molecule, and established a highly sensitive method for detecting this molecule using boron-doped diamond and glassy carbon electrodes by cyclic voltammetry and amperometry.<sup>115</sup> Certainly, electrochemical technique was widely acceptable and popular in detection of *Pseudomonas aeruginosa* and other microorganisms, due to its preponderant feature. For example,

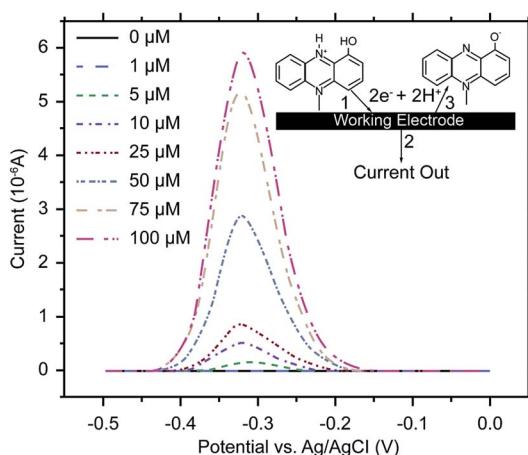


Fig. 4 SWV of human bronchial lavage samples, with pyocyanin added. Scans were performed from 0.5 to 0 V at a frequency of 15 Hz and an amplitude voltage of 50 mV, with the current response from the blank subtracted from each sample measurement. Inset: pyocyanin reacts at the working electrode (1) releasing 2 electrons and 2 hydrogens (2) and is converted to its oxidized form (3). (Adapted from Webster, *et al.*, 2014). Order number from the Copyright Clearance Center: 4066351485823.

Maciej *et al.* (2015) devised a selective electrochemical sensing for human serum albumin (HSA) determination using semi-covalent imprinting, and established an excellent selectivity to the myoglobin and cytochrome c interferences.<sup>116</sup> Sofia *et al.* (2017) reported an electrochemical aptasensor for detection of human osteopontin (OPN), a potential breast cancer biomarker, by using a DNA aptamer selected by SELEX. Their results showed that the aptasensor and the standard ELISA methods quantified similar OPN levels.<sup>117</sup>

## 6. MALDI-TOF MS assays

Matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS) is a new type of soft ionization mass spectrometry, which is used to map the protein spectrum of microorganisms.<sup>118</sup> The mass spectrometry data of clinical microorganisms are compared with the standard protein database of known microorganisms to achieve the purpose of identification.<sup>119</sup> Because of its rapid, accurate, sensitive, automated and high throughput, MALDI-TOF MS has become an efficient microbial rapid identification technology used in clinical diagnosis,<sup>120</sup> environmental monitoring<sup>121</sup> and microbiological classification studies.<sup>122</sup> Some researchers have applied this technique to the identification of *Pseudomonas aeruginosa*. Imperi *et al.* (2009) report a detailed description of the periplasmic proteome of the wild-type *Pseudomonas aeruginosa* strain PAO1 by 2-DE and MALDI-TOF/TOF analysis.<sup>123</sup> In 2012, a rapid detection and identification method for *Pseudomonas aeruginosa* by MALDI-TOF MS was established.<sup>124</sup> It was observed that the results of MALDI-TOF MS for the detection of *Pseudomonas aeruginosa* were consistent with that of automated identification systems Phoenix (BD Biosciences, USA). Recently, MALDI-TOF MS was used to accurately and quickly identify the five high-risk clones of *Pseudomonas aeruginosa* sequence type 111 (ST111), ST175, ST235, ST253, and ST395.<sup>125</sup> More often, this technique was used in *Pseudomonas aeruginosa* drug resistance analysis such as carbapenemase (Wang *et al.*, 2013; Johansson *et al.*, 2014; Hrabak, 2015).<sup>126–128</sup> MALDI-TOF MS represents an innovative technology and has a very good application prospects in the identification of *Pseudomonas aeruginosa*.

MALDI-TOF has been developed for identification of clinical pathogens. In fact, in addition to *Pseudomonas aeruginosa*, many other pathogens were detected using this technique. For example, Camoez *et al.* (2016) described a novel MALDI-TOF-automated method to identify methicillin-resistant *Staphylococcus aureus* (MRSA) lineages from hospital during a 20 year period (1990–2009) and demonstrated that this tool was reliable, powerful, promising for *S. aureus* typing.<sup>129</sup> Maasz *et al.* (2017)<sup>130</sup> reported that MALDI-TOF MS was a cheap, reliable, sensitive and fast detection tool in mayfly and fish species identification and sex determination in bleak (*Alburnus alburnus*).

## 7. Summary and prospect

*Pseudomonas aeruginosa* is a prevalent, opportunistic, Gram-negative bacterium that commonly infects immunocompromised individuals; thus, many cases of hospital-acquired and



community-acquired infections are caused by *Pseudomonas aeruginosa*. *P. aeruginosa* has been associated with increased mortality relative to *Staphylococcus aureus* or other Gram-negative in bloodstream infections.<sup>131</sup> Previous findings have shown that as few as 10–100 bacilli are capable of colonizing the intestine of critically ill or immunocompromised patients.<sup>132</sup> Therefore, early detection of *Pseudomonas aeruginosa* is particularly important. At present, culturing bacteria remains the most commonly applied method for detecting *Pseudomonas aeruginosa* in hospitals, but this method is time-consuming and susceptible to inconsistent results due to sample contamination. In addition, this method has low sensitivity. To address these issues, researchers have developed various assays, each with their own advantages and disadvantages. These methods may be limited by low stability and repeatability with regard to clinical applications, and most importantly, the risk of sample contamination remains unresolved. The developmental trend shows that direct detection of *Pseudomonas aeruginosa* in clinical samples will shorten diagnostic turnaround time and reduce the risk of contamination. In addition, the development of fully enclosed automatic detection techniques likely represents a primary direction of future developments.

## Conflicts of interest

The authors report no conflicts of interest.

## Acknowledgements

We would like to thank Editage ([www.editage.com](http://www.editage.com)) for English language editing. This work was supported by the Scientific Research Fund of the Shenzhen Basic Research Projects under Grant number JCYJ20140901141243646; the GDPRSFS under Grant number 2012; the GDUHTP under Grant number 2011 and 2013; the Science and Technology Planning Project of Guangdong Province under Grant number 2014A020217021; and the Shenzhen Basic Research Projects under (Grant number JCYJ2016043164316142 and JCYJ20170413155047512).

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