

# Identification of commensal *Pseudomonas aeruginosa* isolates using duplex PCR targeting the *oprL* and *algD* genes

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## ABSTRACT

**Background:** *Pseudomonas aeruginosa* (*P. aeruginosa*) is a ubiquitous bacterium that can be found in most moisture places, such as soil, water, food, plants, and animals, including humans. Due to genetic flexibility among strains, there is no standard molecular identification for *P. aeruginosa* from different sources. In this study, monoplex and duplex PCR targeting *oprL*, *algD* and *nfxB* were assessed for the identification of commensal *P. aeruginosa*. **Methods:** Twenty-nine commensal *Pseudomonas* isolates, including 16 *P. aeruginosa* isolates and 13 *P. aeruginosa*-like isolates, were used in the study. First, monoplex PCR targeting *oprL*, *algD* and *nfxB* using published primers was carried out to test their ability to detect commensal *Pseudomonas* isolates. Then, two new primer pairs targeting *oprL* were designed (*oprL*-pp1 and *oprL*-pp2) and used for *oprL*-*algD* duplex PCR to check for the improvement of commensal *P. aeruginosa* detection. **Result and conclusion:** *AlgD* or *nfxB* monoplex PCR had the same sensitivity of 93.75% and specificity of 100%, while *oprL* PCR using published primers was more sensitive (100%) but less specific (0%). Duplex PCR yielded high sensitivity and specificity in detecting *P. aeruginosa*. Both *oprL*-pp1/*algD* and *oprL*-pp2/*algD* duplex-PCR had 93.75% sensitivity (15/16 *P. aeruginosa* isolates) and 100% specificity (0/13 *P. aeruginosa*-like isolates). In addition, *oprL*-pp2 primers were more specific than *oprL*-pp1 primers, with only 2 of 13 *P. aeruginosa*-like isolates detected, while *oprL*-pp1 primers detected all *P. aeruginosa*-like isolates. Compared to the monoplex PCR that only targeted the *oprL* gene, the duplex PCR utilizing *oprL*-pp2 and *algD* primers identified 15/16 *P. aeruginosa* isolates (93.75% specificity). Additionally, the duplex PCR used in this study was negative for non-*Pseudomonas* species, including *E. coli*, *V. cholera*, *V. parahaemolyticus*, and *S. aureus*. In conclusion, our duplex PCR targeting *oprL* and *algD* could be a valuable tool for commensal *P. aeruginosa* screening.

**Key words:** *algD*, duplex PCR, identification, *nfxB*, *oprL*, *Pseudomonas aeruginosa*, sensitivity, specificity

## INTRODUCTION

*Pseudomonas aeruginosa* is a ubiquitous bacterium that can be found in water, soil, food, plants and animals. It can also be isolated from healthy people's skin, throat, and stool and is considered part of the human commensal flora; thus, it is called commensal bacteria<sup>1</sup>. As a serious life-threatening Gram-negative pathogen, they are responsible for a wide range of minor to severe infections in burn patients, immunocompromised individuals or patients with cystic fibrosis. These infections are difficult to eliminate due to the pathogen's intrinsic and extrinsic resistance abilities. Therefore, early and accurate detection of *P. aeruginosa* is critical for treating infected patients.

Many *P. aeruginosa* detection methods have been developed, including traditional bacterial culture methods, immunological assays, and biochemical tests<sup>2</sup>. However, these tests typically take days to weeks to

complete confirmation, with a high rate of misidentification due to cross reactions of *P. aeruginosa* with other related Gram-negative bacilli. In recent years, polymerase chain reaction (PCR) has been developed as a rapid and reliable molecular method for clinical and environmental *P. aeruginosa* detection using a number of specific genes, such as *ecfX*, *gyrB*, *algD*, *oprL*, and *fliC*.<sup>3-5</sup> Multiplex PCR, which allows multiple target detection in a single PCR run, was also considered for clinical *P. aeruginosa*<sup>6</sup>. Commensal isolates, on the other hand, have been investigated even though the detection of these strains is important for tracking and analysis of the pathogen and the spread of antibiotic resistance status<sup>7</sup>. In this study, the application of PCR and multiplex PCR for commensal isolates was investigated.

## MATERIALS AND METHODS

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### Bacterial isolates

Twenty-nine commensal *Pseudomonas* isolates were used in this study. They included 16 *P. aeruginosa* isolates and 11 *P. stutzeri*, 1 *P. azelaica* and 1 *P. nitroreducens* isolates, which were identified via 16S rRNA sequencing (Nam Khoa Biotek CO., Ltd).

### DNA extraction

Bacterial DNA was extracted by the organic phenol–chloroform method<sup>8</sup> and quantitated using Take3 microvolume plates (Synergy HT, Biotek). For long-term storage, DNA was stored in 1X TE (Tris-EDTA) buffer at -20°C.

### Primer design

Two primer sets, *oprL*-pp1 and *oprL*-pp2 (Table 1), were designed based on the *oprL* gene sequence from NCBI [GenBank: 882991] using Primer-Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and Primer3 (<https://primer3.ut.ee/>). An oligo analyzer (<https://www.idtdna.com/calc/analyzer>) was used to check primer quality, including primer dimer and cross-pairing. The melting temperature and annealing temperature of these two primers were set to be similar to the *algD* primer pair. The gradient PCR method was then used to confirm the optimal annealing temperature for newly designed *oprL* primers. Primers were submitted to BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) for comparison with available sequences in the nucleotide database for assessing theoretical *P. aeruginosa* specificity.

### Monoplex and duplex PCR for the detection of *P. aeruginosa*

PCR was carried out in a total reaction volume of 25  $\mu$ L containing 12.5  $\mu$ L master mix (iStandard iVAPCR 2X Master Mix, Viet A Technology Corporation, Vietnam), 2  $\mu$ L DNA template and either 0.5  $\mu$ M of *nfxB* primers, 0.4  $\mu$ M *algD* primers or 0.4  $\mu$ M *oprL* primers (monoplex) or in combination of 0.4  $\mu$ M of each *algD* and *oprL* primer (duplex) (PHUSA Biochem Ltd., Vietnam). PCR was carried out as described in Table 1. PCR products were checked on a 2% agarose gel run at 100 V for 45 minutes in sodium borate (SB) buffer.

### Data analysis

The sensitivity and specificity were determined by the formula from Rajul Parikj MS<sup>9</sup>. In short, sensitivity was calculated as true positive/(true positive+ false negative), and specificity was calculated as true negative/(true negative+ false positive). The data were analyzed using Excel (Microsoft Office, USA).

## RESULTS

### Monoplex PCR using *oprL*-pp0 detected all commensal *Pseudomonas* isolates but not *Pseudomonas aeruginosa* specifically

PCR using *oprL*-pp0 primer pairs detected all 16 *P. aeruginosa* isolates; thus, the sensitivity was 100%. However, it failed to differentiate *P. aeruginosa* isolates from other *Pseudomonas* species, including *P. stutzeri*, *P. azelaica* and *P. nitroreducens*, resulting in a specificity of 0% (Figure 1, Table 2).

### Monoplex PCR targeting *algD* and *nfxB* detected *Pseudomonas aeruginosa* specifically

PCR using *algD* and PCR using *nfxB* gave identical results. All non-*P. aeruginosa* samples were negative. All *P. aeruginosa* isolates gave positive results except one *P. aeruginosa* isolate (isolate 234.1) (Figures 2 and 3). The specificity of PCR using *algD* and *nfxB* for detecting *P. aeruginosa* was 100%, while the sensitivity was 93.75%.

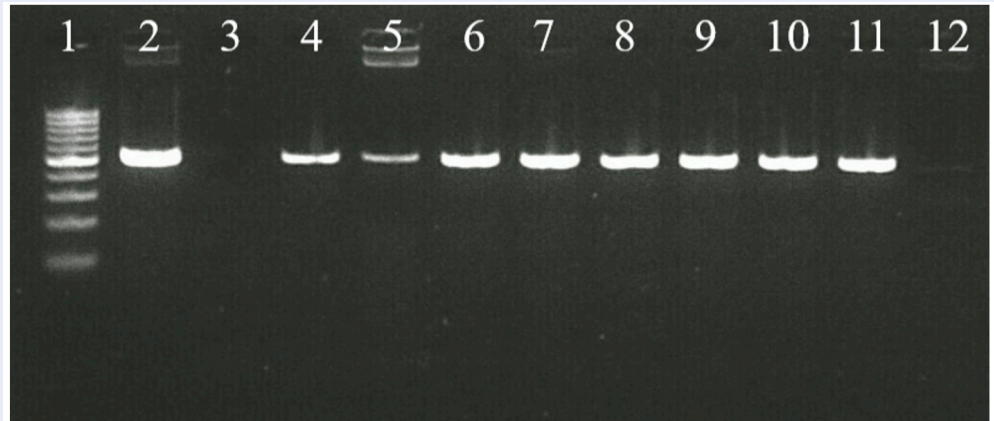
### Duplex PCR targeting *oprL* and *algD*

Two primer pairs for *oprL* were designed in this study with the aim of maintaining the ability to detect 234.1 but not other non-PA isolates. They were coupled with *algD* in a duplex PCR to design an effective identification method for commensal *P. aeruginosa*.

The results showed that *oprL*-pp1 still detected 13/13 non-*P. aeruginosa* isolates, while *oprL*-pp2 could differentiate some of the non-*P. aeruginosa* isolates. In addition, both primer pairs in the *oprL*/*algD* duplex were negative for all other bacterial species (*E. coli*, *S. aureus*, *V. paraheamolyticus*, *V. cholera*) (Table 2).

Both the *oprL*-pp2/*algD* and *oprL*-pp1/*algD* duplex PCR tests had the same detection efficiency, with 100% specificity and 93.75% sensitivity, since there was one isolate (isolate 234.1) detected by *oprL* primers but not by *algD* (Figures 4 and 5). The isolate 234.1 showed only one band for *oprL* in the electrophoresis gel in both duplex PCR assays.

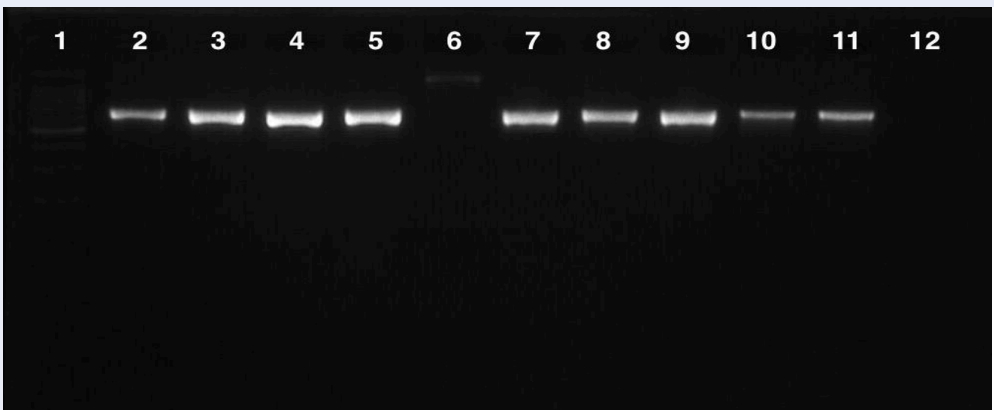
More importantly, duplex PCR using *oprL*-pp2 primers, while maintaining 100% sensitivity to detect *P. aeruginosa*, such as PCR using *oprL*-pp0 and pp1 primer pairs, has a specificity of 84.62%, as only 2/13 *P. aeruginosa*-like isolates gave positive results (Table 2). On the other hand, all other *P. aeruginosa*-like species tested positive using the *oprL*-pp1 primer, similar to the *oprL*-pp0 primers (Table 2), which resulted in 0% specificity. Thus, duplex PCR using *oprL*-pp2/*algD* identified 15/16 *P. aeruginosa* isolates



**Figure 1: Representative PCR products of the oprL gene (oprL-pp0; 504 bp) in Pseudomonas aeruginosa.** Lane 1: 100 bp DNA ladder; lane 2: *P. aeruginosa* ATCC 9027; lane 3: Negative control; lane 4 – 11: samples with positive results; lane 12: sample with negative results.



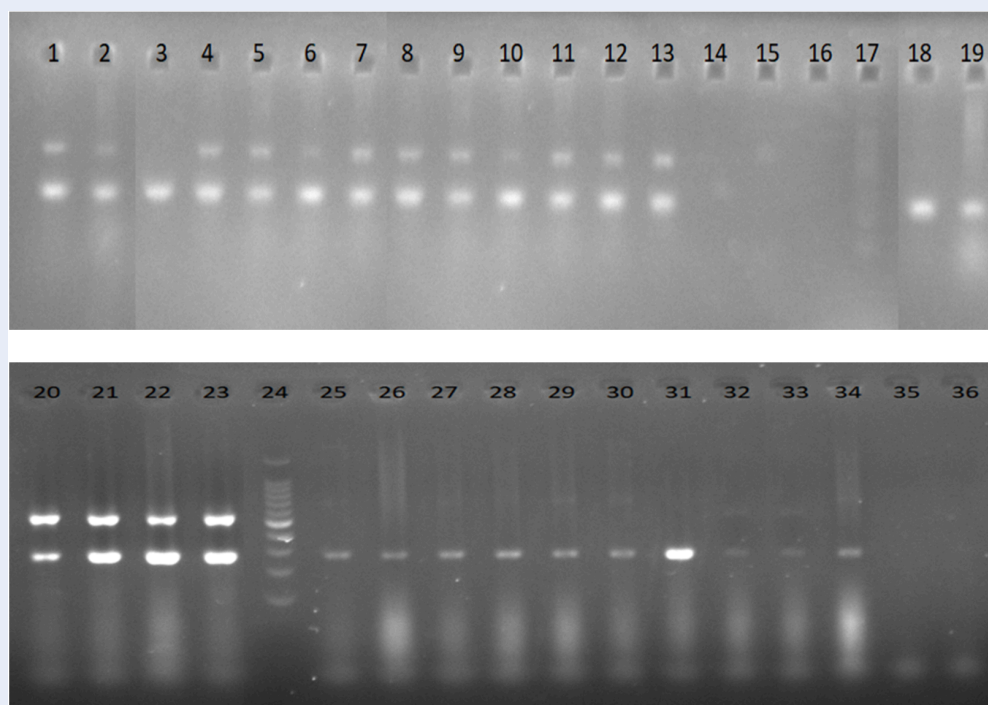
**Figure 2: Representative PCR products of algD gene amplicons (520 bp).** Lane 1: 100 bp DNA ladder; lane 2: *P. aeruginosa* ATCC 9027; lanes 3, 6, 8, 9, 11, 12: *P. aeruginosa* isolates; lanes 4, 5, 7, 10: non-*P. aeruginosa* isolates; lane 13: *E. coli* (negative control); lane 14: non-DNA (negative control).



**Figure 3: Representative PCR products of nfxB gene amplicons (673 bp).** Lane 1: 100 bp DNA ladder; Lane 2: *P. aeruginosa* ATCC 9027; Lanes 3, 4, 5, 7, 8, 9, 10, 11: *P. aeruginosa* isolates; Lane 6: non-*P. aeruginosa* isolates; Lane 12: Negative control.

**Table 1: Characteristics of published and designed primers and nucleotide sequences**

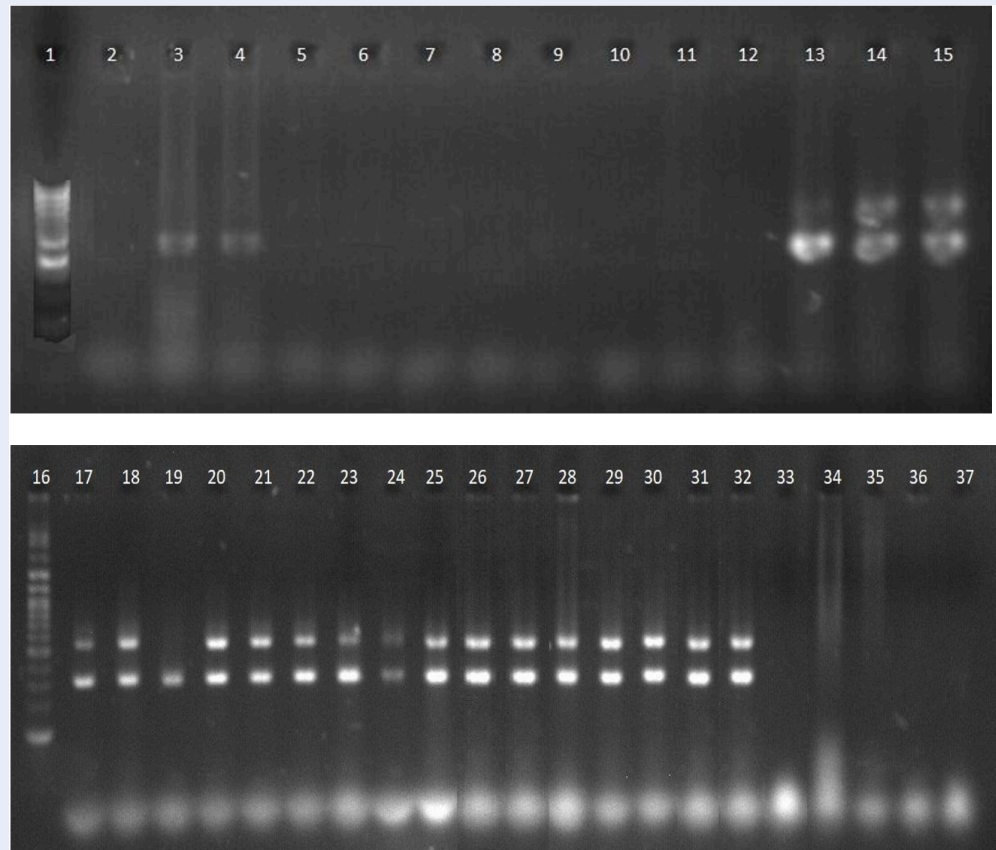
Primers	Oligonucleotide sequence (5' to 3')	PCR program	Target	Primer Length (bp)	Amplicon Size (bp)	Reference
<i>oprL-pp0-F</i>	5'-ATGGAATGCTGAAATTCGGC-3'	30 cycles: 2 min 95°C, 30 s 56°C, 1 min 72°C	<i>oprL</i> gene	21	504	9
<i>oprL-pp0-R</i>	5'-CTTCTTCAGCTCGACGGGACG-3'					
<i>algD-F</i>	5' TTCCCTCGCAGAGAAAACATC 3'	30 cycles: 1 min 94°C, 1 min 60°C, 1 min 72°C	<i>algD</i> gene	21	520	8
<i>algD-R</i>	5' CCTGGTTGATCAGGTCGATCT 3'					
<i>nfxB-F</i>	5'-CGATCCTTCCATTGCACGC-3'	30 cycles: 1 min 94°C, 45 s 49.3°C, 2 min 72°C	<i>nfxB</i> gene	20	673	10
<i>nfxB-R</i>	5'-AGGGTGATGAACAGTTCGGT-3'					
<i>oprL-pp1-F</i>	5'-GCGATCACCCCTTCTACTT-3'	30 cycles: 1 min 95°C, 1 min 60°C, 1 min 72°C	<i>oprL</i> gene	20	258	This study
<i>oprL-pp1-R</i>	5'-CGGACGCTTTACCATAGG-3'					
<i>oprL-pp2-F</i>	5'-CTGTGGGTTGCTCCTCCAAG-3'	30 cycles: 1 min 95°C, 1 min 60°C, 1 min 72°C	<i>oprL</i> gene	20	299	This study
<i>oprL-pp2-R</i>	5'-CTCGCCAGAGCCATAITGT-3'					



**Figure 4: Duplex PCR using *oprL-pp1* (258 bp) and *algD* (520 bp) primers.** Set A (above) and set B (below) performed PCR at the same time. Set A: Lane 1: *P. aeruginosa* (ATCC 9027); lanes 2-13: confirmed *P. aeruginosa* isolates; lanes 18 and 19: non-*P. aeruginosa* isolates; lane 14: *V. parahaemolyticus* (negative control); lane 15: *V. cholerae* (negative control); lane 16: master mix without DNA extract (negative control); lane 17: 100 bp DNA ladder. Set B: Lanes 20-23: confirmed *P. aeruginosa* isolates; lanes 25-34: non-*P. aeruginosa* isolates; lane 35: *E. coli* (negative control); lane 36: *S. aureus* (negative control); lane 24: 100 bp DNA ladder.

**Table 2: Specificity and sensitivity of *oprL/algD* duplex PCR to detect *P. aeruginosa***

PCR	Duplex <i>oprL-pp2/algD</i>		Duplex <i>oprL-pp1/algD</i>		Monoplex
	<i>oprL-pp2</i>	<i>algD</i>	<i>oprL-pp1</i>	<i>algD</i>	
Primer	<i>oprL-pp2</i>	<i>algD</i>	<i>oprL-pp1</i>	<i>algD</i>	<i>oprL-pp0</i>
Confirmed <i>P. aeruginosa</i>	16/16	15/16	16/16	15/16	16/16
Liked <i>P. aeruginosa</i>	2/13	0/13	13/13	0/13	13/13
<i>E. coli</i>	0/1	0/1	0/1	0/1	0/1
<i>S. aureus</i>	0/1	0/1	0/1	0/1	0/1
<i>V. parahaemolyticus</i>	0/1	0/1	0/1	0/1	0/1
<i>V. cholerae</i>	0/1	0/1	0/1	0/1	0/1
Sensitivity	100%	93.75%	100%	93.75%	100%
Specificity	84.62%	100%	0%	100%	0%



**Figure 5: Duplex PCR using *oprL*-pp2 (299 bp) and *algD* (520 bp) primers.** Set A (above) and set B (below) were subjected to PCR at the same time. Set A: Lane 1: 100 bp DNA ladder; lanes 13-15: confirmed *P. aeruginosa* isolates; lanes 2-12: non-*P. aeruginosa* isolates. Set B: Lane 16: 100 bp DNA ladder lane 17: *P. aeruginosa* (ATCC 9027); lane 18-32: *P. aeruginosa* isolates lane 33: *V. parahaemolyticus* (negative control); lane 34: *V. cholerae* (negative control); lane 35: *E. coli* (negative control); lane 36: *S. aureus* (negative control); lane 37: master mix without DNA extract (negative control).

(93.75%) and narrowed the number of *oprL*-positive *P. aeruginosa*-like isolates needing further identification by other methods.

## DISCUSSION

Many studies have found that the PCR approach has a higher sensitivity than the classic culture approach in detecting *P. aeruginosa*, especially at the beginning of colonization<sup>10,11</sup>. Along with the advancement of PCR tests for *P. aeruginosa*, a number of exclusive genes have been found. Khan and Cerniglia developed the first PCR technique for detecting *P. aeruginosa* based on the exotoxin A gene<sup>11</sup>. *oprL*, *oprI*, and *algD* genes and other genes were also considered in monoplex or multiplex PCR tests for *P. aeruginosa* identification<sup>4,12,13</sup>.

*algD* and *oprL* are two exclusive genes that have been used in monoplex PCR to detect *P. aeruginosa* with

high specificity and sensitivity. While the sensitivity of both genes was greater than 90%, the specificity of *algD* was 100% and only slightly higher than 80% for *oprL*<sup>2</sup>. *algD* encodes for GDP — mannose 6 — dehydrogenase of the alginate synthesis pathway<sup>14</sup>, while the *oprL* gene encodes for an outer membrane protein that plays important roles in the interaction of this pathogen with the environment<sup>15</sup>. *nfxB*, a repressor of the MexCD-*oprJ* efflux pump, is another potential target for *P. aeruginosa* detection<sup>16,17</sup>. In this study, the monoplex results showed that *nfxB* maintained the specificity of *algD* but did not improve sensitivity. Both monoplex PCRs targeting the *algD* and *nfxB* genes failed to detect 100% of the *P. aeruginosa* isolates. PCR targeting only a single fragment of the gene results in a lack of precision, as clinical *P. aeruginosa* strains display high genotypic variability<sup>18</sup>. In fact, several studies have reported the absence of one

or more virulence genes in certain strains of *P. aeruginosa*<sup>19,20</sup>.

Multiplex PCR is more efficient than monoplex due to its ability to simultaneously amplify multiple PCR products in a single reaction, allowing for multiplex detection and greatly reducing the cost and time requirements. Aghamollaei et al. developed a *P. aeruginosa* detection assay using triplex PCR that amplifies the *lasI*, *lasR*, and *gyrB* genes, which successfully identified 100% of the clinical isolates tested<sup>6</sup>. However, the specificity of this multiplex PCR against *P. aeruginosa*-like isolates was not fully considered. Another PCR assay using the same genes was effective in identifying 95% of *P. aeruginosa* isolates from Dorper sheep milk<sup>21</sup>. Multiplex PCR can also be developed to allow for more in-depth diagnostics, as multiple bands and single bands can be interpreted differently, minimizing false positives and false negatives<sup>22</sup>.

This study considers the possibility of a duplex PCR detection method using *oprL* coupled with *algD* or *nfxB* genes. PCR targeting the *algD* or *nfxB* genes has a sensitivity of 93.75% and specificity of 100%, while *oprL* is more sensitive but less specific. Although the De Vos study demonstrated that the published *oprL*-pp0 primers could sufficiently detect *P. aeruginosa* from other *Pseudomonas* species<sup>8</sup>, our data suggested that the primer pairs also detect non-*P. aeruginosa* species. In a previous report, the same *oprL*-specific primer set also had just 70% specificity, with only 49 out of 70 *oprL*-positive clinical samples being *P. aeruginosa*<sup>4</sup>. Improvement of the specificity of the *oprL* primers might improve the specificity of this PCR assay. Duplex PCR of *oprL/algD* resulted in 2 bands, indicating confirmed *P. aeruginosa* isolates, while one *oprL* band indicated that further identification methods were needed to confirm the isolate identity, and no band was non-*P. aeruginosa*. At the same time, by designing primers with the same annealing temperature as *algD* or *nfxB*, we can develop a cost-effective duplex PCR to rapidly detect *P. aeruginosa*.

The designed *oprL*-pp2 primer pair in this study was more specific in *P. aeruginosa* detection than *oprL*-pp1 and *oprL*-pp0, with only 2 false-positive results (2/13), while the *oprL*-pp1 primer pair gave positive results for 13/13 *P. aeruginosa*-like isolates (Table 2). With the *oprL*-pp2/*algD* duplex, the number of suspected *P. aeruginosa* isolates (*oprL*-positive only samples) was reduced to a minimum, reducing the amount of further testing. Thus, duplex PCR with *oprL*-pp2 and *algD* had improved specificity in detecting *P. aeruginosa*.

*OprL*-pp2/*algD* duplex PCR is a simple, specific, and sensitive method for *P. aeruginosa* identification. It is

a cost-effective and time-saving method because the processing time from sample preparation to confirmation completion is less than 3 hours. Although *oprL*-pp1/*algD* and *oprL*-pp2/*algD* duplex PCR have the same sensitivity and specificity, the latter produced more precise results since non-*P. aeruginosa* samples were less likely to be *oprL* positive. The 2-target system of the assay decreases the potential for sequence-related false negatives and can provide simultaneous confirmation of positive results.

## CONCLUSION

In conclusion, the results of this study showed that, using the newly designed primers, the duplex PCR assay targeting the *oprL* and *algD* genes was able to identify *P. aeruginosa* with improved specificity. Although additional confirmation of the accuracy of this approach is needed, the results imply that the PCR test presented in this article is a simple, rapid and sensitive tool for the early identification of commensal *P. aeruginosa* isolates.

## LIST OF ABBREVIATIONS

*P. aeruginosa*: *Pseudomonas aeruginosa*  
*P. stutzeri*: *Pseudomonas stutzeri*  
*P. nitroreducens*: *Pseudomonas nitroreducens*  
*V. cholerae*: *Vibrio cholerae*  
*V. parahaemolyticus*: *Vibrio parahaemolyticus*  
*E. coli*: *Escherichia coli*  
*S. aureus*: *Staphylococcus aureus*  
 PCR: Polymerase chain reaction  
 TE: Tris-EDTA  
 SB: Sodium borate

## COMPETING INTERESTS

The authors declare that they have no competing interests.

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## AUTHOR'S CONTRIBUTIONS

Thuc Quyen Huynh wrote the first draft; Nguyen Bao Vy Tran performed the experiments; Thi Thuy Vy Pham collected the isolates; Nguyen Huong Giang Vo performed the experiments; Lam Que Anh Nguyen collected the isolates; Ngoc My Huong Nguyen collected the isolates; Van Dung Nguyen performed the experiment; Thi Thu Hoai Nguyen designed, supervised and reviewed the work.

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