**In-silico** Specificity Comparison between GMF-GMR and JMF-JMR Primers for Detecting moaC Genes of Food Spoilage Bacteria *Pseudomonas* spp.

S N Ethica¹,², A R Sulistyaningtyas¹, S Darmawati¹

¹Faculty of Nursing and Health Sciences, Universitas Muhammadiyah Semarang, Jalan Kedungundu Semarang 18, Semarang, Indonesia, 55273

²Indonesia Forestry Institute (IFI) or Yayasan Kehutanan Indonesia (YKI), Kalibata, Jakarta, Indonesia, 12750

Corresponding author: norma@unimus.ac.id

**Abstract.** *Pseudomonas* spp. have been known as notorious food spoilage bacteria with ability to produce thermo-tolerant enzymes. They pose serious risk to public health as its most pathogenic member, *P. aeruginosa*, could cause nosocomial infections affecting people with immunodeficiency. The use of GMF-GMR primers had been reported capable for detecting bacterial moaC of *Alcaligenes javaensis* JG3. The gene is suspected to be related with dormancy of pathogenic bacteria. This study aimed to investigate specificity of the GMR-GMF as well as a newly designed JMF-JMR pairs of primers (JMF: 5’-GGCGTACATCATCCACACTG-3’ and JMR: 5’-GGCGTTGACCATCTATGACA-3’) for detecting moaC genes of 57 members of *Pseudomonas* spp. retrieved from http://insilico.ehu.eus/ database using in silico PCR (Polymerase Chain Reaction). The results showed that GMF-GMR primers could selectively amplify 271-bp in silico PCR products from 14 out of 57 members of *Pseudomonas* spp. tested. However, BLASTn analysis on these 14 amplified DNA sequences showed that they were not part of moaC, yet glpK gene fragment sequences. Meanwhile, the newly designed primers from moaC sequence of strain JG3, JMF-JMR, could specifically amplify 214-bp in silico PCR products from 2 out of 57 members of *Pseudomonas* spp. matched to bacterial moaC gene fragment sequences. As conclusion, based on in silico study JMF-JMR primers are more specific than GMF-GMR ones for detecting moaC gene fragments of members of *Pseudomonas* spp. studied.

1. Introduction

Food spoilage by *Pseudomonas* spp. in particular food groups such as fish, meat, milk and dairy products, water, fruit, and vegetables. Despite the fact that *Pseudomonas* spp. do not cause serious risk to public health, most pathogenic *Pseudomonas aeruginosa* belongs to risk group II. It is categorized as opportunistic pathogen, which causes nosocomial infections in hospitals affecting immunocompromised persons [1].

Characterization of moaC of food-borne bacterium *Alcaligenes* sp. JG3, later known as *Alcaligenes javaensis* JG3 using degenerate GMF-GMR primers had been reported. Previous result showed that strain JG3 has analogous moaC gene sequence, which is responsible for the synthesis of
MoaC (molybdenum cofactor biosynthesis protein C) of MoaC superfamily involved in molybdenum cofactor (MoCo) biosynthesis similar to that belongs to *Pseudomonas stutzeri* LMG 11199 [2,3]. It is therefore interesting to reveal possible relationship between *moaC* with *Pseudomonads*.

The term *in silico* was coined at the end of the 1980s, to refer to “virtual” experiments existing only “inside” computers. It complements the terms in vivo and in vitro, characterizing experiments that are accomplished, respectively, within a living [4]. In genomic studies, *in silico* PCR is aimed to calculate theoretical PCR results by using up-to-date sequenced bacterial genomes, a technique which allows amplification of specific DNA sequences [4,5]. *In silico* or virtual PCR could be used to predict or to calculate theoretical ability of pairs of primers to amplify targeted gene fragments by using up-to-date sequenced bacterial genomes stored in a database. It is a technique, which allows amplification of specific DNA sequences supporting successful DNA amplification using *in vivo* PCR [5-8]. Today, various web tools for *in silico* PCR including virtual oligonucleotide assembly and analysis have been well-developed. Currently, *in silico* approach is capable to reveal potential PCR biases in the use of DNA barcode. For example, it divulges such biases in the use of internal transcribed spacer (ITS) of nuclear DNA, which has been used as an environmental DNA barcode for fungi for 15 years. In *silico* analysis has been use to investigate virulence genes in an emerging dental pathogen *A. baumannii* and related species. In addition, successful determination of a real-time PCR (Q-PCR) based *in silico* amplification of signature genes for quantification of various bacteria causing sepsis has also been reported [9-11].

Biochemical and molecular methods aiming to detect and confirm the presence of *Pseudomonas* spp. had been studied. However, none of these studies targeting the *moaC* gene sequence of *Pseudomonas* group, while previous report had inferred the link between *moaC* with the food-spoilage agent [2]. This study compared the specificity detection between GMF-GMR and JMF-JMR primers in detecting *moaC* genes of food-spoilage bacterial group of *Pseudomonas* spp. by *in silico* PCR method.

2. Methods

2.1. GMF-GMR primer preparation

GMF-GMR primers were obtained from previous publication [2]. The primers were reported to be able to amplify *moaC* gene fragment of *Alcaligenes javaensis* JG3 using conventional PCR method.

2.2. JMF-JMR Primer design

A pair of gene specific primers were designed using Primer3Plus web-based tool, which was freely available from [http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi](http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) [11]. A DNA template used to design the primers was a *moaC* gene fragment of *Alcaligenes javaensis* JG3 previously reported [2,3]. The sequence was accessed through Genbank accession code of AB894422.1 available from NCBI (National Center for Biotechnology Information) website [https://www.ncbi.nlm.nih.gov/nuccore/AB894422.1?report=fasta](https://www.ncbi.nlm.nih.gov/nuccore/AB894422.1?report=fasta).

2.2.1. *In silico* PCR

An amplification was performed using in silico PCR amplification software freely available from [http://insilico.ehu.es/PCR/](http://insilico.ehu.es/PCR/) (ver. 2010, released by CONSOLIDER-INGENIO, Spain) using GMF-GMR and JMF-JMR primers [6,8]. As many as 57 genomic DNA sequences of members of *Pseudomonas* spp. retrieved from in silico database were used as virtual templates. The genomic sequences of members of *Pseudomonas* spp. encompass notable species from genus *Pseudomonas* such as *P. brassicacearum*, *P. denitrificans*, *P. entomophila*, *P. fluorescens*, *P. fulva*, *P. mendocina*, *P. monteilii*, *P. pose*, *P. protegens*, *P. resinosorans*, *P. aeruginosa*, and *P. syringae*. Separately, both pair of primers were used to amplify the set bacterial genomic DNA sequences. The parameters set included: Exclude plasmid (unchecked), option “allow 1-2 mismatches, but in nucleotides in 3’ end”,

2
and maximum length of band 3000 nucleotides. The amplified products from amplification using both pairs of primers were then analysed using BLASTn from NCBI website [12], and the results were compared based on specificity of products.

3. Results and Discussion

Sequence of moaC previously reported [2] as seen in Figure 1 was retrieved using GenBank accession number: AB894422.1

![Figure 1. DNA sequence of moaC gene fragment of A. javaensis JG3 under (Genbank acc. Code AB894422.1) [Ethica et al., 2018].](image)

From the moaC sequence (Figure 1), a new pair of primers were designed, JMF: 5’-GGCGTACATCATCCACACTG-3’ and JMR: 5’-GGCGTTGACCATCTATGACA-3’ could be designed using Primer3Plus web tool (Figure 2).

![Figure 2. Newly designed primers, JMF (forward) and JMF (reverse) primers obtained from Primer3Plus primer design tool based on moaC sequence of A. javaensis JG3 (blue- and yellow- highlighted).](image)

These newly designed pair of primers, along with the available GMF-GMR primers were used to amplify targeted sequence with 57 genome sequences of Pseudomonas spp. (Table 1) as templates.
Table 1. Genome sequences of 57 members of *Pseudomonas* spp. available from database used as templates of *in silico* PCR to evaluate specificity of GMF-GMR and newly designed IMF-JMR of primers for detection of moaC.

| No. | NCBI Reference Sequence | Genome of Species | Author |
|-----|-------------------------|--------------------|--------|
| 1   | NC_002516.2             | *P. aeruginosa*    | Winsor et al., 2018 |
| 2   | CP004061.1)             | *P. aeruginosa* B136-33 | Lo et al., 2017 |
| 3   | NC_018080.1             | *P. aeruginosa* DK2 | Rau et al., 2017 |
| 4   | NC_002516.2             | *P. aeruginosa* LES431 | Jeukens et al., 2017 |
| 5   | NC_011770.1             | *P. aeruginosa* LESB58 | Winstanley et al., 2017 |
| 6   | NC_017548.1             | *P. aeruginosa* M18 | Wu et al., 2017 |
| 7   | NC_023019.1             | *P. aeruginosa* MTB-1 | Ohshuto et al., 2017 |
| 8   | AP012280.1              | *P. aeruginosa* NGCM2.51 | Miyoshi-Akiyama et al., 2017 |
| 9   | NC_022808.2             | *P. aeruginosa* PAI | Lu et al., 2017 |
| 10  | NC_022806.1             | *P. aeruginosa* PAIR | Le et al., 2017 |
| 11  | NC_009656.1             | *P. aeruginosa* PA7 | Roy et al., 2018 |
| 12  | NC_022594.1             | *P. aeruginosa* PAO1-VE13 | Yin et al., 2017 |
| 13  | NC_022591.1             | *P. aeruginosa* PAO1-VE2 | Yin et al., 2017 |
| 14  | NC_022361.1             | *P. aeruginosa* PAO581 | Yin et al., 2017 |
| 15  | NC_021577.1             | *P. aeruginosa* RP73 | Jeukens et al., 2017 |
| 16  | NC_023149.1             | *P. aeruginosa* SCV20265 | Eckweiller et al., 2017 |
| 17  | NC_008463.1             | *P. aeruginosa* UCBPP-PA14 | Lee et al., 2017 |
| 18  | NC_022360.1             | *P. aeruginosa* e7447m | Yin et al., 2017 |
| 19  | NC_015379.1             | *P. brassicacearum* NFSM421 | Oreet et al., 2017 |
| 20  | CP004143.1              | *P. denitrificans* ATCC 13867 | Aimala et al., 2014 |
| 21  | NC_008027.1             | *P. entomophila* L48 | Vodovar et al., 2018 |
| 22  | NC_017911.1             | *P. fluorescens* A506 | Loper et al., 2018 |
| 23  | NC_016830.1             | *P. fluorescens* F113 | Redondo-Nieto et al., 2017 |
| 24  | NC_004129.6             | *P. fluorescens* F5 | Paulsen et al., 2017 |
| 25  | NC_007492.2             | *P. fluorescens* P10 | Silby et al., 2017 |
| 26  | NC_012660.1             | *P. fluorescens* SBW25 | Silby et al., 2017 |
| 27  | CP002727.1              | *P. fulva* 12-X | Lucas et al., 2011 |
| 28  | CP002620.1              | *P. mendocina* NK-01 | Guo et al., 2014 |
| 29  | NC_009439.1             | *P. mendocina* ymp | Copeland et al., 2017 |
| 30  | NC_023075.1             | *P. monteilii* SB3078 | Dueholm et al., 2017 |
| 31  | NC_023076.1             | *P. monteilii* SB3101 | Dueholm et al., 2017 |
| 32  | NC_020209.1             | *P. poae* RE*1-1-14 | Mueller et al., 2017 |
| 33  | CP003190.1              | *P. protegens* CHA0 | Schuldes et al., 2014 |
| 34  | CP002290.1              | *P. putida* BIRD-1 | Matilla et al., 2014 |
| 35  | CP003734.1              | *P. putida* DOT-TIE | Matilla et al., 2014 |
| 36  | CP000712.1              | *P. putida* F1 | Copeland et al., 2017 |
| 37  | CP000926.1              | *P. putida* GB1 | Copeland et al., 2017 |
| 38  | CP005976.1              | *P. putida* H8234 | Molina et al., 2014 |
| 39  | CP003738.1              | *P. putida* H83267 | Molina et al., 2015 |
| 40  | NC_002947.4             | *P. putida* KT2440 | Belda et al., 2016 |
| 41  | NC_021505.1             | *P. putida* NRBC 14164 | Ohji et al., 2018 |
| 42  | NC_017986.1             | *P. putida* ND6 | Li et al., 2018 |
| 43  | NC_015733.1             | *P. putida* S16 | Yu et al., 2017 |
| 44  | EU514690.1              | *P. putida* UW4 | Cheng et al., 2010 |
| 45  | NC_010501.1             | *P. putida* W619 | Copeland et al., 2017 |
| 46  | AP013068.1              | *P. resinovorans* NBRC 106553 | Shintani et al., 2016 |
| 47  | NC_023064.1             | *Pseudomonas* sp. TKP | Ohshuto et al., 2017 |
| 48  | NC_022738.1             | *Pseudomonas* sp. YL120 | Volmer et al., 2017 |
| 49  | NC_009434.1             | *P. stutzeri* A1501 | Yan et al., 2017 |
| 50  | CP002881.1              | *P. stutzeri* LMG 11199 | Chen et al., 2016 |
| 51  | NC_018028.1             | *P. stutzeri* CCUG 29243 | Brunet-Galvez et al., 2017 |
| 52  | CP003725.1              | *P. stutzeri* DSM 10701 | Busquets et al., 2015 |
| 53  | CP002622.1              | *P. stutzeri* DSM 4166 | Yu et al., 2015 |
| 54  | CP003071.1              | *P. stutzeri* RCH2 | Lucas et al., 2013 |
| 55  | JPMV10000039.1          | *P. syringae*      | Baltrus et al., 2014 |
| 56  | CP000581.1              | *P. syringae* pv. phaseolicola 1448A | Joarda et al., 2017 |
| 57  | NC_007005.1             | *P. syringae* pv. syringae B728a | Feil et al., 2016 |
All parameters set on \textit{in silico} PCR using JMF-JMR primers could be seen in Figure 3.

\begin{center}
\textbf{Figure 3.} \textit{In silico} PCR using newly designed primers, JMF (forward) and JMF (reverse) primers obtained from Primer3Plus primer design tool based on \textit{moaC} sequence of \textit{A. javaensis} JG3.
\end{center}

\begin{center}
\textbf{Figure 4.} Products of \textit{in silico} PCR amplification using a pair of GMF-GMR primers with genomic DNA sequences of 57 members \textit{Pseudomonas} spp. as template, which were retrieved from \textit{in silico} database (http://insilico.ehu.es/) (Bikandi, 2004). Single bands of 271-bp DNA matched to \textit{glpK} gene fragments were obtained from 14 members of \textit{Pseudomonas} spp., which consists of \textit{Pseudomonas aeruginosa} strains represented by lanes: 1. \textit{P. aeruginosa} sp., 2. \textit{P. aeruginosa B136-33}, 3. \textit{P. aeruginosa} DK2 4. \textit{P. aeruginosa LES431} 5. \textit{P. aeruginosa LEB58} 6. \textit{P. aeruginosa M18} 7. \textit{P. aeruginosa MTB-1} 8. \textit{P. aeruginosa NCGM2.S19}. \textit{P. aeruginosa PA110}. \textit{P. aeruginosa PAIR11}. \textit{P.
aeruginosa PA 15. P. aeruginosa RP73 16. P. aeruginosa SCV20265 17. P. aeruginosa UCBPP-PA14.

Meanwhile, results of in silico PCR using both GMR-GMF as well as JMF-JMR pairs of primers are shown in Figure 4 and Figure 5, respectively. As seen in Figure 4, the amplified virtual PCR products are single bands sized ~271 bp. These results showed that GMF-GMR primers could selectively amplify ~271-bp in silico PCR products from 14 out of 57 genomes of members of Pseudomonas spp. tested. However, BLASTn analysis on these 14 amplified DNA sequences showed that all of them were not part of moaC, yet glpK gene fragment sequences. These results were not in line with conventional PCR using similar primers GMR-GMF with genomic DNA of Alcaligenes javaensis JG3 as template. As previously reported, DNA sequence of PCR product obtained from conventional PCR using GMF and GMR on DNA genome of strain JG3 resulted moaC gene fragment with assigned Genbank accession code of AB894422.1 [2]. Instead, the obtained moaC sequence, was then used to design new primers JMF-JMR reported in this study.

Meanwhile, the newly designed primers from moaC sequence of strain JG3, JMF-JMR, could specifically amplify 214-bp in silico PCR products from 2 out of 57 members of Pseudomonas spp. DNA sequences of these PCR products were matched to moaC gene fragment sequences belong to two strains of P. stutzeri species. Results of in silico PCR using JMF-JMR primers with 57 genome sequences of Pseudomonas spp. are shown in Figure 5.

As seen in Figure 5, lanes number 49 and 53 showed single bands sized ~214 bp. These lanes belong to P. stutzeri A1501 and P. stutzeri DSM 4166 genomic sequences, respectively. BLASTn analysis on these PCR products showed that both DNA sequences shared 100% similarity with moaC gene fragments of P. stutzeri A1501 (Figure 6) and P. stutzeri DSM 4166 (Figure 7), also with similar gene of Alcaligenes javaensis JG3 [3].

Conserved domains of both predicted protein sequences displayed in Figure 6 and Figure 7 have been reported in NCBI as cyclic pyranopterin monophosphate synthase (MoaC, Molybdenum Cofactor Biosynthesis Protein C). Based on information from protein data bank (PDB), MoaC is a
member of the MoaC superfamily cI00242, which hypothetical structure is displayed in Figure 8. Members of cI00242 superfamily are involved in molybdenum cofactor (Moco) biosynthesis, an essential cofactor of a diverse group of redox enzymes. MoaC, a small hexameric protein, converts, together with MoaA, a guanosine derivative to the precursor Z by inserting the carbon-8 of the purine between the 2’ and 3’ ribose carbon atoms initiating three phases of Moco biosynthesis [14-16].

**Pseudomonas stutzeri A1501**
>NC_009434, from 1284772 to 1284985 (214 bp); Pseudomonas stutzeri A1501
GGCGTTGACCATCTATGACATGTGCAAGGCCGTTGACCGCGCATGTTGATCAGAAGGGTGCAGCTGC
TGAAAGGTGGCGCAAATGGACATGTGCAAGGCCGTTGACCGCGCATGTTGATCAGAAGGGTGCAGCTGC
GTATCGGGAAAACCTAGCCAGCGATGCGAGCGCATCAATGGGATGAATCGTTCGGCAGTGTGGAT
GATGTACGCC

Identical protein: Cyclic pyranopterin monophosphate synthase MoaC [Pseudomonas stutzeri] NCBI Reference Sequence: WP_011912355.1
>WP_011912355.1 cyclic pyranopterin monophosphate synthase MoaC [Pseudomonas stutzeri]
MLTHLDSLGRASMVDVTDKAVTAREAVAEARVRMLPQTLQLQQGGHPKGVDVFAVARIAIQAKKTH
ELIPLCHPPITTSSKVELQADGEDSVLIRAVCKLAGQTGVEMEALTAASVAALTITYDCKAVDRGNYI
EGVRRLKGGKSGHQQVQA

**Figure 8.** Hypothetic structure of MoaC cI00242 superfamily which members include MoaC and MoaA proteins (Source: Protein Data Bank, PDB).
Boutros and Okey reported that the information in a primer pair is combined by an in-silico PCR to identify potential amplicons by both identity and size [17]. In silico PCR results allow the user to accept or reject potential primer pairs for experimental use. Based on this, the newly designed pair of primers JMF-JMR obtained in this study were theoretically accepted and were potential to proceed for in vitro PCR use to detect moaC of P. stutzeri from Pseudomonas spp. JMF-JMR showed theoretically potential uses to detect the occurrence of P. stutzeri contamination on foods based on its moaC genes amplified by these primers.

4. Conclusion

Based on in silico study, JMF-JMR primers are more specific than GMF-GMR ones for detecting moaC genes fragment of 57 members of Pseudomonas spp. studied, which include strains of species P. brassicaevarum, P. denitrificans, P. entomophila, P. fluorescens, P. fulva, P. mendocina, P. monteilii, P. pose, P. protegens, P. resinovorans, and P. syringae. As conclusion, based on in silico study, JMF-JMR primers are more specific than GMF-GMR ones for detecting moaC genes fragment of members of Pseudomonas spp. studied.

5. Author Contribution Statement

Dr. SNE contributed in preparing and analyzing all research data reported. ARS contributed in preparing manuscript and summarizing research data, Dr. SD contributed in final editing the manuscript.

References

[1] Raposo, A., Pérez, E., de Faria, C. T., Ferrús, M. A., & Carrascosa, C. (2016). Food Spoilage by Pseudomonas spp.—An Overview. Food Borne Pathogens and Antibiotic Resistance, 241.

[2] Ethica, S. N., Semiarti, E., Widada, J., Oedjijono, O., & Joko Raharjo, T. (2017). Characterization of moaC and a non-target gene fragments of food-borne pathogen Alcaligenes sp. JG3 using degenerate colony and arbitrary PCRs. Journal of Food Safety, 37(4), e12345.

[3] Ethica, S. N., Oedjijono, O., Semiarti, E., Widada, J., & Raharjo, T. J. (2018). Genotypic and Pheno typic Characterization of Alcaligenes javaensis JG3 Potential as an Effective Biodegrader. BIOTROPIA-The Southeast Asian Journal of Tropical Biology, 25(1), 1-10.

[4] Moretti, S. (2011). In silico experiments in scientific papers on molecular biology. Science & Technology Studies.

[5] Kalendar, R., Muterkö, A., Shamekova, M., & Zhambak, K. (2017). In Silico PCR Tools for a Fast Primer, Probe, and Advanced Searching. In PCR (pp. 1-31). Springer, New York, NY.

[6] Bikandi, J., Millán, R. S., Rementeria, A., & Garaizar, J. (2004). In silico analysis of complete bacterial genomes: PCR, AFLP–PCR and endonuclease restriction. Bioinformatics, 20(5), 798-799.

[7] Ethica, S. N., Hammi, M. K., Lestari, P., Semiarti, E., Widada, J., & Raharjo, T. J. (2013). Amplification of Azospirillum sp. JG3 glpD gene fragment using degenerate primers generated by web-based tools. The Journal of Microbiology, Biotechnology and Food Sciences, 3(3), 231.

[8] Ethica, S. N., & Raharjo, T. J. (2014). Detection of genes involved in glycerol metabolism of Alcaligenes sp. JG3 (Doctoral dissertation, Universitas Gadjah Mada).

[9] Priyadharsini, J. V., Girija, A. S., & Paramasivam, A. (2018). In silico analysis of virulence genes in an emerging dental pathogen A. baumannii and related species. Archives of oral biology, 94, 93-98.
[10] Gupta, S., Dongre, A., Saxena, J., & Jyoti, A. (2017). Computation and in silico validation of a real-time PCR array for quantitative detection of pathogens isolated from blood sample in sepsis patients.

[11] Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P., & Kauserud, H. (2010). ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. *BMC microbiology, 10*(1), 189.

[12] Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., & Leunissen, J. A. (2007). Primer3Plus, an enhanced web interface to Primer3. *Nucleic acids research, 35*(suppl_2), W71-W74.

[13] Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., & Madden, T. L. (2008). NCBI BLAST: a better web interface. *Nucleic acids research, 36*(suppl_2), W5-W9.

[14] Schwarz, G., & Mendel, R. R. (2006). Molybdenum cofactor biosynthesis and molybdenum enzymes. *Annu. Rev. Plant Biol., 57*, 623-647.

[15] Wuebbens, M. M., Liu, M. T., Rajagopalan, K. V., & Schindelin, H. (2000). Insights into molybdenum cofactor deficiency provided by the crystal structure of the molybdenum cofactor biosynthesis protein MoaC. *Structure, 8*(7), 709-718.

[16] Schwarz, G. (2005). Molybdenum cofactor biosynthesis and deficiency. *Cellular and Molecular Life Sciences CMLS, 62*(23), 2792-2810.

[17] Boutros, P. C., & Okey, A. B. (2004). PUNS: transcriptomic-and genomic-in silico PCR for enhanced primer design. *Bioinformatics, 20*(15), 2399-2400.