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DETECTION OF VIRULENCE GENES *phoP* AND *phoQ* IN *Salmonella* spp. USING *IN SILICO* POLYMERASE CHAIN REACTION

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ABSTRACT

Detection of Salmonella bacteria based on their virulence genes is among essential steps in the eradication of clinical infection by bacteria. In this study, two pair of primers, PhoPF-PhoPR: 5'-CCGCGCAGGAAAAACTCAAA-3' and 5'-ATCTGTTCCAGCATCACCGG -3' as well as PhoQF-PhoQR: 5'-AGAGATGATGCGCGTACTGG-3' and 5'- CAGACGCCCCATGAGAACAT-3', had been successfully designed using Primer3Plus to detect the presence of phoP and phoQ genes in Salmonella spp. Using genomic DNA of 44 genomic data of Salmonella spp. as templates, PhoPF-PhoPR could produce 520-bp amplicon, while PhoQF-PhoQR could result in 598-bp amplicon. Results of in silico PCR showed that both pairs of primers PhoPF-PhoPR and PhoQF-PhoQR could detect only Salmonella enterica species, and no Salmonella bongori species could be detected based on phoP and phoQ sequences. Both pairs of PhoPF-PhoPR and PhoQF-PhoQR primers were also able to detect the virulence genes in most of the studied subspecies of Salmonella enterica available in silico database unless Arizona subspecies. As conclusion, based on this in silico study, phoP and phoQ genes appeared to be biomarkers for Salmonella enterica species. Both pairs of primers designed in this study has potential to be used as detection tool to differentiate species Salmonella enterica from Salmonella bongori, and also to distinguish S.enterica subsp. enterica from subsp. Arizonae.
***Keywords**: Gene detection, bacterial virulence, phoP, phoQ, Salmonella spp.*

ABSTRAK

Deteksi bakteri *Salmonella* berdasarkan gen virulensinya adalah salah suatu langkah penting dalam pemberantasan infeksi klinis oleh bakteri. Dalam kajian ini, dua pasang primer, PhoPF-PhoPR: 5'-CCGCGCAGGAAAAACTCAAA-3' dan 5' ATCTGTTCCAGCATCACCGG-3' serta PhoQF-PhoQR: 5'-AGAGATGATGCGCGTACTGG-3' dan 5'- CAGACGCCCCATGAGAACAT-3'telah berhasil dirancang menggunakan program Primer3Plus untuk mendeteksi keberadaan gen *phoP* dan *phoQ* pada grup *Salmonella* spp. Menggunakan DNA genom dari 44 bakteri *Salmonella* spp. sebagai cetakan, PhoPF-PhoPR dapat menghasilkan amplikon berukuran 520 bp, sedangkan PhoQF-PhoQR dapat menghasilkan amplikon berukuran 598 bp. Hasil *in silico* PCR menunjukkan bahwa kedua pasang primer, PhoPF-PhoPR dan PhoQF-PhoQR, hanya mendeteksi spesies *Salmonella enterica*, namun tidak mendeteksi spesies *Salmonella bongori* berdasarkan sekuen gen *phoP* dan *phoQ*. Primer PhoPF-PhoPR dan PhoPF-PhoPR mampu mendeteksi gen virulensi *phoP* and *phoQ* pada semua strain *S.enterica* subspesies *enterica* yang terdapat dalam *database* dengan pengecualian subspesies Arizonae. Sebagai kesimpulan, berdasarkan studi *in silico*, gen *phoP* dan *phoQ* terlihat sebagai biomarka bagi spesies *S.enterica*. Kedua pasang primer yang dirancang dalam penelitian ini memiliki potensi untuk digunakan sebagai alat deteksi yang membedakan spesies *S.enterica* dari *S.bongori* dan juga memisahkan *S.enterica* subsp. enterica dari subsp. Arizonae.
Kata kunci: Deteksi gen, virulensi bakteri, *phoP*, *phoQ*, *Salmonella* spp.

INTRODUCTION

Salmonella infections are common and can lead in protean clinical manifestations, which ranges from an asymptomatic condition to highly severe disease. In the United States only, infection by the non-Typhi salmonellae causes an estimated 1.4 million illnesses per year, leading to an estimated 15,000 hospitalizations and more than 400 deaths (Voetsch et al., 2004). Salmonella serotypes are closely related genetically yet differ significantly in their pathogenic potentials. Understanding the mechanisms responsible for this may be key to a more general understanding of the invasiveness of intestinal bacterial infections (Jones *et al*., 2008).

Salmonellae belong to the family Enterobacteriaceae and are a medically important pathogen for both humans and animals. Salmonellae form a complex group of bacteria consisting of two species and six subspecies and al lot of serovars (Andino and Hanning, 2015). Only two species are currently recognized in the genus Salmonella, *S. enterica* and *S. bongori* (Tyndall et al., 2005). Differentiating infection by *S.enterica* from *S.bongori* is important to help in a more targeted clinical treatment or medication due to Salmonella infection.

Specific and unique sequence of DNA which marks the presence of particular bacterium could be used as bacterial biomarker. Molecular detection of virulence genes as markers bacteria has been widely done. For example, molecular detection of virulence genes *toxA*, *aprA*, *rhlAB*, *plcH*, *lasB* and *fliC* in *Pseudomonas aeruginosa* isolated from urinary tract infections and these genes are known as markers of this bacterium (Sabharwal *et al*., 2014). The *phoP* and *phoQ* are among virulence genes belong to Salmonella (Søborg *et al*., 2016). PhoP is an essential transcription factor for bacterial virulence (Ren *et al*., 2019). Along with *phoP*, phoQ is a response regulator in two-component regulatory system. The phoP/phoQ regulon enables *S.Typhimurium* to adapt to intramacrophage stresses other than phagolysosomal fusion (Thompson *et al*., 2011).

Detection of genes using Polymerase Chain Reaction (PCR) requires specific sequences known as primers. A primer serves as the starting point for DNA replication, specific binding of the oligonucleotide to the target sequence on the template strand is essential for a successful experiment. The binding specificity of a primer is determined by several of its properties, like the melting temperature (Tm), GC-content and self-complementarity. Designing primers is usually done with the help of computer programs, among which Primer3 is among the most widely used. Primer3Plus is an enhanced web interface to Primer3 (Untergasser *et al*., 2007).

In silico analysis is among important bioinformatics tools to obtain specific genes of virulence bacteria. *In silico* analysis has been use to investigate virulence genes in an emerging dental pathogen *A. baumannii* and related species. *In silico* amplification of signature genes for quantification of many sepsis causing bacteria had also been reported (Priyadharsini *et al*., 2018; Gupta *et al*., 2017). An *in silico* approach is also beneficial in revealing potential PCR biases

(Bellemain *et al*., 2010). This study aimed to design specific primers using Primer3Plus that can be used to detect the presence of virulence biomarker of Salmonella species, the *phoP* and *phoQ* genes, using in *silico PCR*. Products of *in silico* PCR were analyzed using BLASTn, a nucleotide-nucleotide search to align similar sequences and calculated the homology levels. This work was also intended to confirm that *phoP* and *phoQ* genes are genetical markers for *S.enterica*.

METHOD

Primers PhoPR dan PhoPF were designed from virulence transcriptional regulatory protein PHOP of the genome of *Salmonella enterica* subsp. *enterica* ser. Typhimurium str. LT2, Gene symbol: *phoP*; Gene description: Response regulator in two-component regulatory system with PhoQ; Locus tag: STM1231; Gene type: protein coding; Gene ID: 1252749, Genomic Sequence: NC_003197.2; Gene product="response regulator in two-component regulatory system with PhoQ"; Sequence: NC_003197.2 (1318679..1319365, complement).

Primers PhoQF dan PhoQR were designed from virulence transcriptional regulatory protein PHOQ of the genome of *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain LT2, Gene symbol: *phoQ*; Gene description: response regulator in two-component regulatory system with PhoQ; Locus tag: STM1231; Gene type: protein coding; Gene ID: 1252749, Genomic Sequence: NC_003197.2; Gene product="response regulator in two-component regulatory system with PhoQ"; Seq.: NC_003197.2 (1317216..1318679, complement).

Primer design work was done using Primer3Plus (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) followed by *in silico* PCR available at http://insilico.ehu.eus (San Millán *et al*., 2013; Bikandi *et al*., 2004; Untergasser *et al*., 2012; Ethica *et al*., 2019) Result analysis included PCR product size and BLASTn (Chen *et al*., 2015; Ethica, 2014) to confirm that the amplicons were truly part of *phoP* and *phoQ* gene sequence

Table 1. List of *Salmonella* spp. which genomic DNA used as in silico PCR templates

List of genomic DNA of *Salmonella* spp. used as templates of *in silico* PCR	
1. *Salmonella bongori* N268-08	23. *Salmonella enterica* subsp. *enterica* Paratyphi A str. ATCC 9150
2. *Salmonella bongori* NCTC 12419	24. *Salmonella enterica* subsp. *enterica* Paratyphi B str. SPB7
3. *Salmonella enterica* subsp. *arizonae* serovar 62:z4,z23:--	25. *Salmonella enterica* subsp. *enterica* Paratyphi C strain RKS4594
4. *Salmonella enterica* subsp. *enterica* Cubana str. CFSAN002050	26. *Salmonella enterica* subsp. *enterica* Pullorum str. S06004
5. *Salmonella enterica* subsp. *enterica* Agona str. 24249	27. *Salmonella enterica* subsp. *enterica* Schwarzengrund str. CVM19633
6. *Salmonella enterica* subsp. *enterica* Agona str. SL483	28. *Salmonella enterica* subsp. *enterica* Thompson str. RM6836
7. *Salmonella enterica* subsp. *enterica* Bareilly str. CFSAN000189	29. *Salmonella enterica* subsp. *enterica* Typhi
8. *Salmonella enterica* subsp. *enterica* Bovismorbificans str. 3114	30. *Salmonella enterica* subsp. *enterica* Typhi Ty2
9. *Salmonella enterica* subsp. *enterica* Choleraesuis str. SC-B67	31. *Salmonella enterica* subsp. *enterica* Typhi str. P-stx-12
10. *Salmonella enterica* subsp. *enterica* Dublin str. CT_02021853	32. *Salmonella enterica* subsp. *enterica* Typhi str. Ty21a
11. *Salmonella enterica* subsp. *enterica* Enteritidis str. P125109	33. *Salmonella enterica* subsp. *enterica* Typhimurium DT104
12. *Salmonella enterica* subsp. *enterica* Gallinarum str. 287/91	34. *Salmonella enterica* subsp. *enterica* Typhimurium LT2
13. *Salmonella enterica* subsp. *enterica* Pullorum str. CDC1983-67	35. *Salmonella enterica* subsp. *enterica* Typhimurium str. 08-1736
14. *Salmonella enterica* subsp. *enterica* Pullorum str. RKS5078	36. *Salmonella enterica* subsp. *enterica* Typhimurium str. 14028S
15. *Salmonella enterica* subsp. *enterica* Heidelberg str. 41578	37. *Salmonella enterica* subsp. *enterica* Typhimurium str. 798
16. *Salmonella enterica* subsp. *enterica* Heidelberg str. B182	38. *Salmonella enterica* subsp. *enterica* Typhimurium str. D23580
17. *Salmonella enterica* subsp. *enterica* Heidelberg str. CFSAN002069	39. *Salmonella enterica* subsp. *enterica* Typhimurium str. DT2
18. *Salmonella enterica* subsp. *enterica* Heidelberg str. SL476	40. *Salmonella enterica* subsp. *enterica* Typhimurium str. SL1344
19. *Salmonella enterica* subsp. *enterica* Javiana str. CFSAN001992	41. *Salmonella enterica* subsp. *enterica* Typhimurium str. ST4/74
20. *Salmonella enterica* subsp. *enterica* Newport str. SL254	42. *Salmonella enterica* subsp. *enterica* Typhimurium str. T000240
21. *Salmonella enterica* subsp. *enterica* Newport str. USMARC-S3124.1	43. *Salmonella enterica* subsp. *enterica* Typhimurium str. U288
22. *Salmonella enterica* subsp. *enterica* Paratyphi A str.AKU_12601	44. *Salmonella enterica* subsp. *enterica* Typhimurium str. UK-1

Note: Different species and subspecies from *Salmonella enterica* are highlighted in bold

RESULTS AND DISCUSSION

Features of a number of important but poorly explained human clinical syndromes strongly indicate a microbial etiology. In these syndromes, the failure of cultivation-dependent microbial detection methods reveals our ignorance of microbial growth requirements. Sequence-based molecular methods, however, offer alternative approaches for microbial identification directly from host specimens found in the setting of unexplained acute illnesses, chronic inflammatory disease, and from anatomic sites that contain commensal microflora (Relman, 1998).

In this study, *in silico* PCR as one of sequence-based molecular methods was used to help in fast identification of *Salmonella enterica* from *Salmonella* spp. samples. Pair of primers were developed as the basis for the detection of *Salmonella enterica*. using *in silico* PCR as predictive tool prior to *in vitro* PCR (Ethica et al., 2013). The search of unique virulence gene *phoP* and *phoQ* belong to *S.enterica* subsp. *enterica* was previously carried out using NCBI *search tool*. PCR primer design was carried out based on sequences of these unique gene. Next, primers design followed by *in silico* PCR was done.

Primer Design

DNA sequences of *phoP* obtained from NCBI GenBank database was used as basis of primer design of is shown in **Figure 1,** while its 3-dimension protein structure (SWISSMODEL) is illustrated in **Figure 2**. Another one, the *phoP* DNA sequence is shown in **Figure 3**, while its protein structure (3-dimension SWISSMODEL) is displayed in **Figure 4**. Fasta format of the sequence was copied and then used as input in Primer3Plus web-based tool.

Salmonella enterica subsp. *enterica* serovar Typhimurium str. LT2, complete genome NCBI Reference Sequence: NC_003197.2 (*phoP*)

```
>NC_003197.2:c1318679-1317216 Salmonella enterica subsp. enterica serovar Typhimurium str.
LT2, complete genome
ATGAATAAATTTGCTCGCCATTTTCTGCCGCTGTCGCTGCGGGTTCGTTTTTTGCTGGCGACAGCCGGCG
TCGTGCTGGTGCTTTCTTTGGCATATGGCATAGTGGCGCTGGTCGGCTATAGCGTAAGTTTTGATAAAAC
CACCTTTCGTTTGCTGCGCGGCGAAAGCAACCTGTTTTATACCCTCGCCAAATGGGAAAATAATAAAATC
AGCGTTGAGCTGCCTGAAAATCTGGACATGCAAAGCCCGACCATGACGCTGATTTACGATGAAACGGGCA
AATTATTATGGACGCAGCGCAACATTCCCTGGCTGATTAAAAGCATTCAACCGGAATGGTTAAAAACGAA
CGGCTTCCATGAAATTGAAACCAACGTAGACGCCACCAGCACGCTGTTGAGCGAAGACCATTCCGCGCAG
GAAAAACTCAAAGAAGTACGTGAAGATGACGATGATGCCGAGATGACCCACTCGGTAGCGGTAAATATTT
ATCCTGCCACGGCGCGGATGCCGCAGTTAACCATCGTGGTGGTCGATACCATTCCGATAGAACTAAAACG
CTCCTATATGGTGTGGAGCTGGTTCGTATACGTGCTGGCCGCCAATTTACTGTTAGTCATTCCTTTACTG
TGGATCGCCGCCTGGTGGAGCTTACGCCCTATCGAGGCGCTGGCGCGGGAAGTCCGCGAGCTTGAAGATC
ATCACCGCGAAATGCTCAATCCGGAGACGACGCGTGAGCTGACCAGCCTTGTGCGCAACCTTAATCAACT
GCTCAAAAGCGAGCGTGAACGTTATAACAAATACCGCACGACCCTGACCGACCTGACGCACAGTTTAAAA
ACGCCGCTCGCGGTTTTGCAGAGTACGTTACGCTCTTTACGCAACGAAAAGATGAGCGTCAGCAAAGCTG
AACCGGTGATGCTGGAACAGATCAGCCGGATTTCCCAGCAGATCGGCTATTATCTGCATCGCGCCAGTAT
GCGCGGTAGCGGCGTGTTGTTAAGCCGCGAACTGCATCCCGTCGCGCCGTTGTTAGATAACCTGATTTCT
GCGCTAAATAAAGTTTATCAGCGTAAAGGGGTGAATATCAGTATGGATATTTCACCAGAAATCAGTTTTG
TCGGCGAGCAAAACGACTTTGTCGAAGTGATGGGCAACGTACTGGACAACGCTTGTAAATATTGTCTGGA
GTTTGTCGAGATTTCGGCTCGCCAGACCGACGATCATTTGCATATTTTCGTCGAAGATGACGGCCCAGGC
ATTCCCCACAGCAAACGTTCCCTGGTGTTTGATCGCGGTCAGCGCGCCGATACCCTACGACCAGGACAAG
GCGTGGGGCTGGCTGTCGCGCGCGAGATTACGGAACAATACGCCGGGCAGATCATTGCCAGCGACAGTCT
GCTCGGTGGCGCCCGTATGGAGGTCGTTTTTGGCCGACAGCATCCCACACAGAAAGAGGAATAA
```

Figure 1. Sequence of *phoP* gene retrieved from GenBank

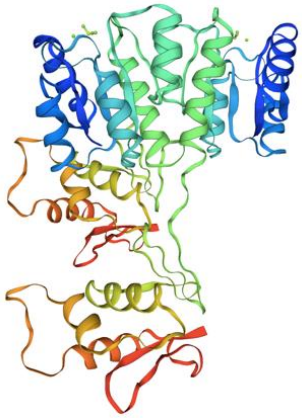

Figure 2. Structure of virulence transcriptional regulatory protein (PhoP) according to Swiss Model (UniProtKB accession code P0DM78)

Salmonella enterica subsp. enterica serovar Typhimurium str. LT2, complete genome
NCBI Reference Sequence: NC_003197.2 (*phoQ*)

```
>NC_003197.2:c1319365-1318679 Salmonella enterica subsp. enterica serovar Typhimurium str.
LT2, complete genome
AAGGGAGAAGAGATGATGCGCGTACTGGTTGTAGAGGATAATGCATTATTACGCCACCACCTGAAGGTTC
AGCTCCAGGATTCAGGTCACCAGGTCGATGCCGCAGAAGATGCCAGGGAAGCTGATTACTACCTTAATGA
ACACCTTCCGGATATCGCTATTGTCGATTTAGGTCTGCCGGATGAAGACGGCCTTTCCTTAATACGCCGC
TGGCGCAGCAGTGATGTTTCACTGCCGGTTCTGGTGTTAACCGCGCGCGCGAAGGCTGGCAGGATAAAGTCG
AGGTTCTCAGCTCCGGGGCCGATGACTACGTGACGAAGCCATTCCACATCGAAGAGGTAATGGCGCGTAT
GCAGGCGTTAATGCGCCGTAATAGCGGTCTGGCCTCCCAGGTGATCAACATCCCGCCGTTCCAGGTGGAT
CTCTCACGCCGGGAATTATCCGTCAATGAAGAGGTCATCAAACTCACGGCGTTCGAATACACCATTATGG
AAACGCTTATCCGTAACAACGGTAAAGTGGTCAGCAAAGATTCGCTGATGCTTCAGCTGTATCCGGATGC
GGAACTGCGGGAAAGTCATACCATTGATGTTCTCATGGGGCGTCTGCGGAAAAAAATACAGGCCCAGTAT
CCGCACGATGTCATTACCACCGTACGCGGACAAGGATATCTTTTTGAATTGCGCTAA
```

Figure 3. Sequence of *phoQ* gene retrieved from GenBank

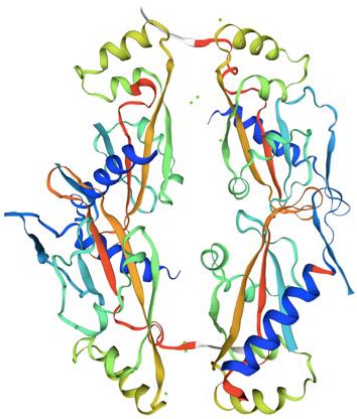

Figure 4. Structure of virulence sensor histidine kinase protein (PhoQ) according to Swiss Model (UniProtKB accession code P0DM80)

PCR Design results from Primer3Plus are shown in **Table 2** Amplicon with the largest possible size and quality of primers (Ethica, 2014; Ethica et al., 2017) were the main consideration to select of the best primers selection in this step. However, it is also importat to make sure that in silico PCR products of these primers are truly part of *phoP* or *phoQ* genes. Based on result in **Table 1**, the selected primers to amplify *phoP* fragment were PhoPF-PhoPR: 5'-CCGCGCAGGAAAAACTCAAA-3' and 5'-ATCTGTTCCAGCATCACCGG-3'. Size of PCR amplicon obtained using these

primers was 520 bp allowing 42 of 44 all Enteric subspecies detected

On the other hand, the selected pair of primers to amplify phoP fragment was PhoQF-PhoQR: 5'-AGAGATGATGCGCGTACTGG -3' and 5'-CAGACGCCCCATGAGAACAT -3'. Both pair of primers were then subjected to *in silico* PCR. The amplicon size using the primers was 598 bp, also allowing all Enteric species detected.

Table 2. Resulted primers designed from phoP and phoQ seguence using Primer3Plus

Input sequnce	Pair of primer code	Forward primer	Reverse primer	*In silico* PCR amplicon size (bp)	Detected sub-species
phoP gene	P1	CCGCGCAGGAAAAACTCAAA	ATCTGTTCCAGCATCACCGG	520	42 of 44
	P2	GGTGGAGCTTACGCCCTATC	CCAGTACGTTGCCCATCACT	522	17 of 44
	P3	TGAAACCAACGTAGACGCCA	AGCTTTGCTGACGCTCATCT	544	27 of 44
	P4	AACAAATACCGCACGACCCT	AATGATCTGCCCGGCGTATT	591	41 of 44
	P5	TCCTTTACTGTGGATCGCCG	TTTGCTCGCCGACAAAACTG	512	40 of 44
phoQ gene	Q1	ATTACGCCACCACCTGAAGG	TGACATCGTGCGGATACTGG	596	40 of 44
	Q2	AGAGATGATGCGCGTACTGG	CAGACGCCCCATGAGAACAT	598	42 of 44
	Q3	TGCGCGTACTGGTTGTAGAG	CGCATCCGGATACAGCTGAA	545	37 of 44
	Q4	GCTCCAGGATTCAGGTCACC	ACGCCCCATGAGAACATCAA	532	40 of 44
	Q5	GGATTCAGGTCACCAGGTCG	GCGGATACTGGGCCTGTATT	557	41 of 44

Note: Selected pair of primers are highlighted in grey

***In Silico* PCR**

Genomic DNA of *Salmonella* spp. stored in the *in silico* PCR database were used as templates. Using 44 genomic DNA of of *Salmonella* spp. as *in silico* PCR templates, primer PhoPF-PhoPR could result in 520-bp amplicon (**Figure 5**). Results of *in silico* PCR showed that PhoPF-PhoPR could be used to differentiate species *S.enterica* from *S.bongori*. The primer was also able to detect the virulence genes in most of serovars of *S.enterica* stored in database unless subspecies Arizonae.

On the other hand, using genomic DNA of 44 serovars of *Salmonella* spp. as templates, primer PhoQF-PhoQR could produce longer length of amplicon, 598 bp (**Figure 6**). Results of *in silico* PCR showed that PhoQF-PhoQR primers were able to detect the virulence genes in most of serovars of *S.enterica* subsp. *enterica* stored in *in silico* database with an exception subspecies Arizonae.

BLASTn analysis had confirmed that *in silico* PCR products were part of either *phoP* or *phoQ* genes. BLASTn is a nucleotide sequence analysis by comparing target DNA sequence of targeted gene or gene fragment to the existed ones available in Genbank (Raharjo et al., 2011).

It was reported that PhoP/PhoQ as a two-component system controls several physiological and virulence functions in *S.enterica*. This system is activated by low Mg^{2+}, acidic pH, and antimicrobial peptides. It was recently found that depending on whether the inducing signal is acidic pH or low Mg^{2+}. PhoP/PhoQ system regulates different Salmonella genes. (Choi et al., 2009).

Based *in silico* study conducted, both *phoP* and *phoQ* genes appeared to be biomarkers for *S.enterica* species. Two pairs of primers designed in this study have potential to be used as detection tool to differentiate species *S.enterica* from *S.bongori*. This study also demonstrated that the primers could also be used as detection tool of all of *S.enterica* subsp. *enterica* leaving only subspecies Arizonae undetected.

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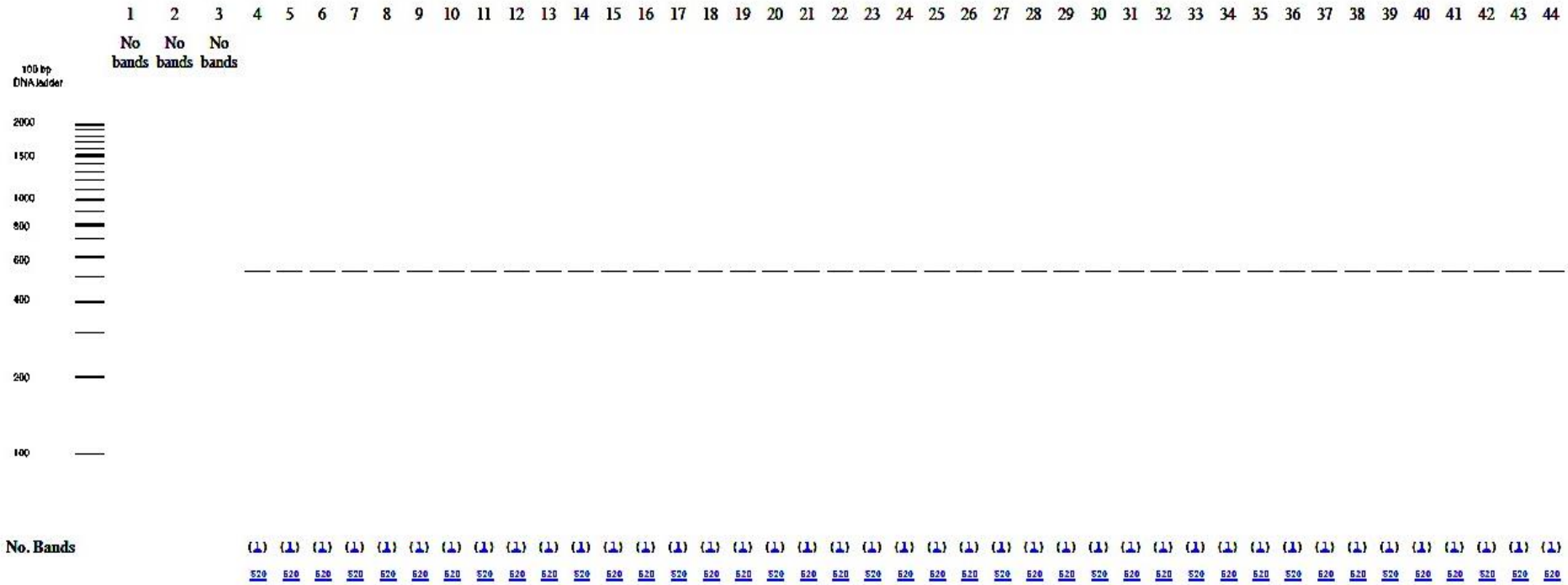

Figure 5. Amplification products of *in silico* PCR (520 bp) using designed primer PHOPF-PHOPR and 44 Salmonella genomic DNA as templates (see **Table 1**)

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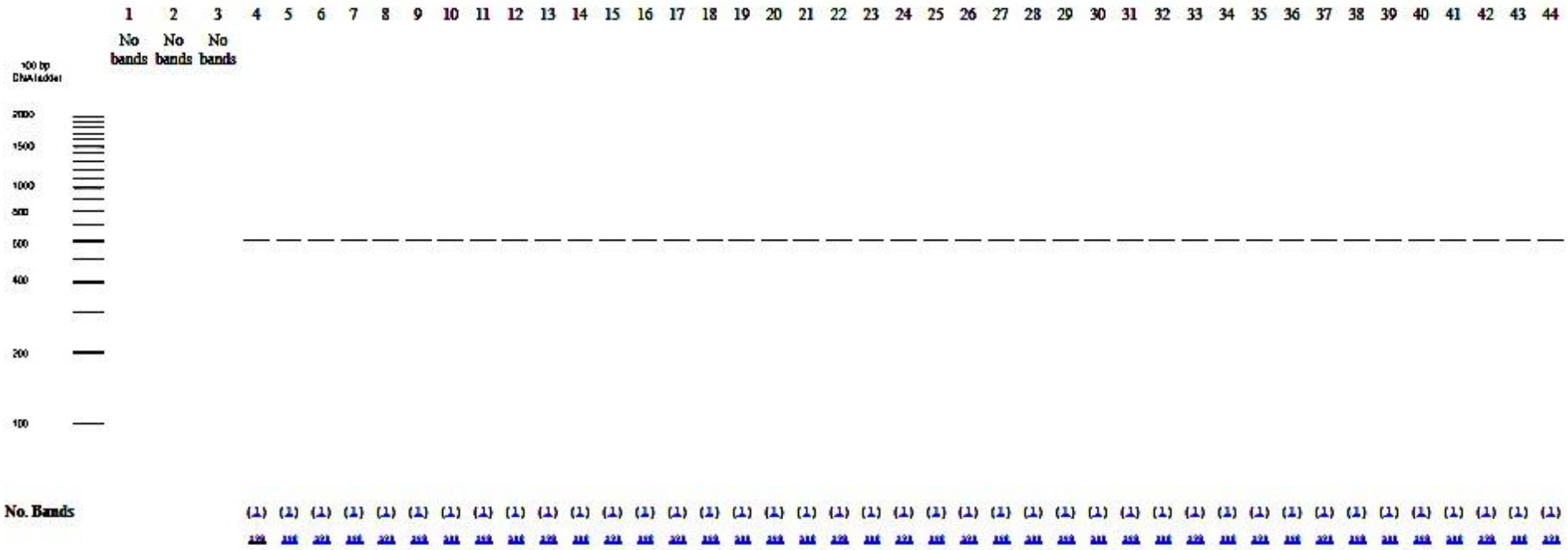

Figure 6. Amplification products of *in silico* PCR (598 bp) using designed primer PHOQF-PHOQR and 44 Salmonella genomic DNA as templates (see **Table 1**)

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Conclusion

based on this *in silico* study, *phoP* and *phoQ* genes appeared to be biomarkers for *S.enterica* species. Both pairs of primers designed in this study has potential to be used as detection tool to differentiate species *S.enterica* from *S.bongori* and also to distinguish *S.enterica* subsp. enterica from subspecies Arizonae.

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