Detection of mutation at Serine-83inQRDR of *gyrA*gene of drug-resistant *Salmonella enterica*serovarTyphiisolates from Kelantan, Malaysia using PCR-RFLP and DNA sequencing

Maizan M[a], C.W. Salma CWZ[a], FadhilahK[c], ZaidahAR[b], PhuaKK[a]
[a]Institute for Research in Molecular Medicine (INFORMM),[b] School of Medical Sciences, UniversitiSains Malaysia, 16150 KubangKerian, Kelantan, Malaysia.[c]Veterinary Faculty, University Malaysia Kelantan, 16100 PengkalanChepa, Kota Bharu,Kelantan, Malaysia.

Corresponding author: Maizan M.,

Abstract

A total of 30 drug-resistant (DR)including 2 multidrug-resistant (MDR) *Salmonellaenterica*serovarTyphi(*S*.Typhi) isolates from Kelantan, Malaysia were examined for the presence of Serine-83(Ser-83) to Phenylalanine(Phe) or Tyrosine (Tyr)substitution in the quinolone resistance-determining region (QRDR) of *gyrA* gene by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Results were further confirmed by sequencing of the amplified products. The *Tfi*1restriction enzyme used in PCR-RFLP cuts only the non-mutant strain of *S*.Typhi. Interestingly, this enzyme cut all the 28ciprofloxacin or nalidixic-resistant *S*.Typhiisolates and MDR S0147/07 isolate that resistant to ampicillin, ciprofloxacin, nalidixic and trimethoprim-sulfamethoxazolebut not the MDR B7377/05 *S*.Typhiisolate which is resistant to chloramphenicol, ampicillin and Trimethoprim-sulfamethoxazole. Sequencing results further confirmed these findings where a point mutation at Ser-83 toPhe(TCC-TTC) in the QRDRof *gyrA* gene was only observed in MDR B7377/05 isolate. These results are in contradictory to the previous findings where most of the ciprofloxacin or nalidixic-resistant isolates have single or double mutationsat Ser-83 or Aspartic-87 (Asp-87)in the QRDR of *gyrA* gene.

KEYWORDS: Salmonella *enterica*serovarTyphi, PCR-RFLP, Sequencing.

INTRODUCTION

Typhoid fever remains as an endemic and global health problem especially in developing countriesdue to lack of personal hygiene and sanitation [10, 27]. World Health Organisation (WHO)has estimated about 16 to 17 million typhoid cases with 600,000 deaths occur every year [8]. The infection is caused by *Salmonella enterica*serovarTyphi[29]. The basis of typhoid fever treatment is an antimicrobial therapy [34].Chloramphenicol was the "gold standard" agent for the treatment of this infection until 1970s [22], but due to the emergence of chloramphenicol-resistant strains, ampicillin and trimethoprim were considered as drugs of choice[9]. However, *S*.Typhi strain resistant to chloramphenicol, ampicillin, and Trimethoprim-sulfamethoxazole(WHO termed as multidrug resistant[MDR])has

emerged in the Indian subcontinent, Southeast Asia and Africa [3,5,8,17,18, 23,27,33]. This has led to the use of ciprofloxacin (fluoroquinolone) and nalidixic(quinolone) as alternative drugsfor the treatment of typhoid fever [15]. However, in the past few years, *S.*Typhi strains resistant to these drugswerealso reported especially in the Asia regions due to the improper use of antibiotics in treating the infectious diseases [4, 6, 19, 30]. In this situation, third generation cephalosporin group of antibiotic (ceftriaxone)is recommended for the treatment of typhoid [16].

The disease is endemic in Malaysia affecting all age groups especially the children [24]. Between years 2001 to 2007, the incidence rates were around 2 to 5 cases per 100,000 populations [31]. The highest incidence was reported in the State of Kelantan in 2005 with more than 800 cases [24]. Currently the occurrence of typhoid fever in Malaysia is sporadic with occasional outbreaks in the areas where sanitary systems are poor [2].

The primary target ofquinolones and fluoroquinolonesactivities are DNA gyraseand topoisomerase IV, which respectivelyencoded by *gyrA* and *gyrB*and*parC* and*parE*genes.Mutations of *gyrA* gene conferring to resistance have been clustered in a region of the gene product between amino acids 67 and 122 termed as QRDR[35]. Mutationat Ser-83 to Phe or Tyrof this regionhas been reported to associate with resistance and decreased susceptibility to ciprofloxacin andresistant to nalidixic acid[1,9,11,14, 15,16,21,26,33,34]. Thus, present study was aimed to determinethe profile of mutation at theQRDR region of *gyrA*gene of the DR*S.*Typhiisolates from Kelantan, Malaysia by PCR-RFLPand further confirmed by DNA sequencing. We hope that this PCR-RFLP methodcould be used forfuture rapid diagnosis of DR S. Typhi isolates from Kelantan, Malaysia.

MATERIALS AND METHODS

Bacterial strains

ThirtyDR *S.*Typhiisolates were obtained from Culture Bank of Institute for Research in Molecular Medicine (INFORMM) and Department ofParasitology and Microbiology, UniversitiSainsMalaysia (USM) from years 2002 to 2009. They were identified as *S.*Typhiisolates by standard bacterial culture and biochemical reaction methods[25]. The isolates were previously tested for susceptibility to chloramphenicol,ampicillin,ciprofloxacin, nalidixicand trimethoprim-sulfamethoxazoleusing a Kirby-Bauer disc diffusion method [13]. Interpretation of the resultswereaccording toClinical and Laboratory Standards Institute(CLSI)guidelines[7]. The susceptibility results were as shown in Table 1 [12].

PCRamplification

DNA extraction from *S.*Typhi colonies was performed using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), according to the protocol provided by the kit manufacturer. Extracted DNA was subjected to amplification by PCR with primers specific for the QRDR of *gyrA* as previously described [15]. Amplification was performed in a thermal cycler (Applied Biosystems, Foster City, CA) using the following protocol: Initial denaturation step at 95° C for 5 min, followed by 30 cycles of 1

min of denaturation at 95° C, 1 min of annealing at 55° C, and 1 min of extension at 72° C, with a final extension step of 5 min at 72° C. PCR products were resolved on 2% agarose gel with ethidium bromide dye and visualized under a UV transilluminator. PCR products were purified using QIAquick spin PCR purification kit (QIAGEN, Hilden, Germany).

RFLP

Restriction maps of the amplified *gyr*A gene sequences were determined by pDRAW32 DNA analysis (AcaClone software).The*Tfi*1 enzyme was found to cutat the nucleotide (TCC) position of Ser-83 in the QRDR of *gyrA*gene of wild type *S.*Typhi strain but not the mutant strain. Digestion reaction was performed by incubating 5 µl of PCR product with 5 U of *Tfi*l restriction enzyme at 65°C for 1 hr in a final reaction volume of 15 µl. The digestion products were resolved on 2% agarose gel in Tris-Borate-EDTA(TBE) buffer and visualized by staining with ethidium bromide. Undigested PCR product was run together in the gel as a control.

Nucleotide Sequencing

To verify the mutation detected by PCR-RFLP, purified PCR products were subjected to sequencing using ABI Prism 3110 genetic analyzer by Big-Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Nucleotide sequences were converted to amino acid sequences using Generunner version 3.05 (Hastings software, Inc.). The amino acid sequences were NCBI blasted and aligned using Bioedit program [20].

Results

PCR amplification

PCR products of all the 30*S.*Typhi isolates used in this study revealed an expected band size corresponding to 331bp. No band was observed in a negative control sample (Figure 1).

Restriction Fragment Length Polymorphism

Restriction digestion product of amplified *gyrA* gene with *Tfi*Ienzyme showsonly MDRB7377/05 isolate remained uncut with 331 bp product (Lane 17), while the PCR product of 29 isolatesincluding MDR S0147/07 isolate were cut into2 fragments corresponding to 140 and 191bp(Figure 2).

Sequencing of QRDR of *gyrA*

Amino acid sequences of the QRDR of *gyrA* gene revealed no mutation in the 29 either ciprofloxacin ornalidixic-resistant*S.*Typhi isolates including the MDR S0147/07 isolate. Only *S.*Typhi MDR B7377/05 isolate showed a point mutation at Ser-83 to Phe(Table 1). No mutation at the Asp-87 was observed in all the 30 DR *S.*Typhiisolates including the MDR B7377/05 and S0147/07.

DISCUSSION

Previous studies have shown that mutation at Ser-83 or Asp-87 in the QRDR of*gyrA* gene is associated with resistance to nalidixic acid or decreased susceptibility to ciprofloxacin[1, 11, 14, 15, 16, 21, 26, 28, 32, 34].This mutation has been suggested as an important determination marker for these resistances. In this study, PCR-RFLP method using *Tfi*Irestriction enzyme targeting the nucleotide sequence of Ser-83 in the QRDR of *gyrA*gene of *S.* Typhi isolates was used. The enzyme cleaves only the PCR product of non-mutant *S.* Typhi isolates. This PCR-RFLP method

has successfully differentiated between the mutant and non-mutant strains of DR S.Typhi isolates from Kelantan, Malaysia. These results were in agreement with the sequencing finding, where only the MDR B7377/05 isolate resistant to chloramphenicol, ampicillin and trimethoprim-sulfamethoxazolehas a single mutation at this region. The other 28 isolates resistant to either nalidixic or ciprofloxacin and MDR S0147/07 isolate resistant to ampicillin, trimethoprim-sulfamethoxazole, ciprofloxacin and nalidixic acid showed no mutation of Ser-83 of *gyrA*gene. It is in contradictory with the previous finding, where most of the nalidixic or ciprofloxacin resistance isolates have mutation at Ser-83 in the QRDR of *gyrA* gene [11, 14, 15, 16, 21, 26, 28, 32, 34]. No mutation was also observed at Asp-87 of this gene for all the 30 Kelantan DR *S*. Typhi isolates including the 2 MDRstrains (B7377/05 and S0147/07). It is possible that for Kelantan DR *S*. Typhi isolates, mutation which contributes to these resistances may be present in other genes and should be further investigated. Interestingly, mutation at Ser-83 was only observed in MDR B7377/05 isolate that is resistant to the ampicillin, trimethoprim-sulfamethoxazoleand chloramphenicol drugs, but not in the S0147/07 isolate that is resistant to all the 4 drugs; ampicillin, trimethoprim-sulfamethoxazole, ciprofloxacin and nalidixic acid, except to the chloramphenicol and ceftriaxonedrugs. Thus, the Ser-83 to Phe mutation in MDR B7377/05 isolate might arise due to the resistance to the chloramphenicol drug. Unfortunately we cannot further confirm this finding since we do not have isolate that is resistant only to chloramphenicol drug. However this method need to be further validated on more MDR and chloramphenicol-resistant Kelantan isolates which are currently unavailable in our CultureBank.

In conclusion, this PCR-RFLP method could be used in identifying the MDR *S*.Typhi Kelantan isolates that are resistant to ampicillin, trimethoprim-sulfamethoxazole and chloramphenicoldrugs but not for the nalidixic or ciprofloxacin-resistant isolates. These results were in contradictory to the previous findings where mutation at this region wasused as a genetic marker for the nalidixic or ciprofloxacin-resistant *S*. Typhistrains.

ACKNOWLEDGEMENTS

This work was funded by Research University Grant 1001/CIPPM/812059 entitled ‘Genetic variation and Molecular epidemiology of *S*.Typhi isolated from Kelantan, Malaysia’.

CONFLICT OF INTEREST STATEMENT

We declare that we do not have conflict of interest.

REFERENCES

1.Afroj, S., Ilias, M., Islam, M., Saha,K.S.(2011) A Prevalence of Ciprofloxacin and Nalidixic Acid Resistant *Salmonella entericas*erovarTyphi in Bangladesh.BangladeshJof Microbiol.28, pp. 7-11.

2.Anita,S.S.,Amir, K.M.,Fadzilah, K.K.,Ahamad, J.J.,Noorhaida,U.U.,Marina, K.K.,Paid,M.Y., Hanif,Z.Z. (2012) Risk factors for typhoid outbreak in Sungai

CongkakRecreational Park, Selangor 2009.Med J Malaysia.67(1), pp. 12-16.

3.Bernard, R., Linda, R.W., Threlfall,E.J. (1997) Multidrug-Resistant *Salmonella*Typhi: A Worldwide Epidemic. Clin Infect Dis. 24(Suppl 1), pp. 106-109

4. Brown, J.C., Shanahan, P.M.A., Jesudason, M.V.,Thomson, C.J.,Amyes,S.G.B. (1996) Mutations responsible for reduced susceptibility to 4-quinolones in clinical isolates of multi-resistant *Salmonella* Typhi in India.J AntimicrobChemother.37, pp. 891-900.

5. Chandrasekaran, B.,Balakrishnan, S. (2011) Screening, phylogenetic analysis and antibiotic sensitivity pattern of *Salmonella enterica*serovarTyphi isolates from typhoid asymptomatic carriers. Asian Pac J Trop Med.4(10), pp. 769-772.

6.Chau, T.T., Campbell, J.I., Galindo, C.M., Van Minh Hoang, N., Diep, T.S., et al. (2007) Antimicrobial drug resistance of *Salmonella enterica*serovarTyphi in Asiaand molecular mechanism of reduced susceptibility to the fluoroquinolones. AntimicrobAgents Chemother.51, pp.4315-4323.

7. Clinical and Laboratory Standards Institute. (2009) Performance Standards for antimicrobial disc susceptibility test. Approved Standard.10th Edition.

8. Crump, J.A., Luby, S.P., Mintz, E.D. (2004) The global burden of typhoid fever.Bull World Health Organ. 82, pp.346-353.

9. Crump, J.A., Mintz, E.D. (2010) Global trends in typhoid and paratyphoid fever. Emerg infect Dis. 50, pp. 241-246.

10. Connor, B.A., Schwartz,E. (2005) Typhoid and paratyphoid fever in travelers. The Lancet Infect Dis.5 (10), pp. 623-628.

11. Dimitrov,T., Dashti,A.A., Albaksami,O., Jadaon, M.M. (2010) Detection of mutations in the *gyr*A gene in fluoroquinolone resistance *Salmonella enterica* serotypes Typhi and Paratyphi A isolated from the Infectious Diseases Hospital, Kuwait. J Clin.Pathol.l63, pp.83-87.

12. DuPont, H. L. (1993)Quinolones in *Salmonella* Typhiinfection.Drugs.45, pp. 119-124.

13. Fadhilah, K. (2010) Molecular analysis of *Salmonella enterica*serovarTyphi isolates using Pulsed-Field Gel Electrophoresis (PFGE). M.Sc. Thesis in Molecular Medicine. Institute for Research in Molecular Medicine (INFORMM), UniversitiSains Malaysia (USM), Malaysia.

14. Gaind, R., Paglietti, B., Murgia, M., Dawar, R., Uzzau, S., Cappuccinelli, P., Deb, M., Aggarwal, P., Rubino, S. Molecular characterization of ciprofloxacin-resistant *Salmonella enterica*serovarTyphi and Paratyphi A causing enteric fever in India.J of AntimicrobChemother.2006; 58: 1139-1144.

15. Griggs, D.J., Gensberg, K., Piddock, L.J. (1996) Mutations in *gyrA* gene of quinolone-resistant *Salmonella* serotypes isolated from humans and animals.

Antimicrob.AgentsChemother.40, pp.1009-1013.

16. Gulati, S., Marwaha, R.K., Singhi, S., Ayyagari, A., Kumar, L. (1992) Third generation cephalosporins in multi-drug resistant typhoid fever. Indian Pediatr.29, pp.513-516.

17. Ling, J., Chang, P.Y. (1984) Plasmid mediating resistance to chloramphenicol, trimethoprim and ampicillin in *Salmonella*Typhi in Southeast Asia regions.J. Infect Dis. 149, pp.652.

18. Kariuki, S., Gilks, C., Revathi, G., Hart, C.A. (2000) Genotypic analysis of multidrug-resistant *Salmonellaenterica*serovarTyphi, Kenya.Emerg Infect Dis. 6(6), pp. 649-651.

19. Kothari, A.,Pruthi, A., Chugh, T.D. (2008) Theburden of enteric fever.J Infect DevCtries.2, pp.253-259.

20. Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment and analysis program for window 95/98/NT. Nuc. Acids Symp.41, pp. 95-98.

21.Hirose, K., Hashimoto, A.I., Tamura, K., Kawamura, Y., Ezaki, T., Sagara, H., Watanabe, H. (2002) DNA Sequence Analysis of DNA Gyrase and DNA Topoisomerase IV Quinolone Resistance-Determining Regions of *Salmonella enterica*serovarTyphi and serovarParatyphi A.Antimicrob Agents Chemother.46(10), pp.3249-3252.

22.Islam, A., Butler, T., Kabir, I., Alam, N.H. (1993) Treatment of typhoid fever with ceftriaxone for 5 days or chloramphenicol for 14 days: a randomized clinical trial. Antimicrob Agents Chemother.37, pp.1572-1575.

23.Jesudason, M.V., Jacob John, T. (1990) Multiresistant*Salmonella* Typhiin India. Lancet, 336, pp.252.

24.Ministry of Health Malaysia.(2007). Incidence of Notifiable Communicable Diseases in Malaysia for 1990-2006. Available online at http://www.dph.gov.my/survelans/Statistik/incidenceofnotifiablecdc90-2006.pdf

25. Murray, P.R., Baron, E.J., Pfaller, M.A., Tenover, F.C., Yolken, R.H. (1999) Manual of clinical microbiology. 7th ed. Washington (DC): American Society for Microbiol. pp 442-458.

26.Nobthai, P., Serichantalergs, O.,Wongstitwilairoong, B., Srijan, A., Bodhidatta, L., Malla, S., Mason, C.J.(2010) Emergence and properties of fluoroquinolone resistant *Salmonella enterica*serovarTyphi strains isolated from Nepal in 2002 and 2003. Southeast Asian J Trop Med Public Health.41(6), pp. 1416-1422.

27.Ochiai, R.L., Acosta, C.J., Danovaro-Holliday, M.C., Baiqing, D., Bhattacharya, S.K., et al.(2008) A study of typhoid fever in five Asian countries: disease burden andimplications for controls. Bull World Health Organ86, pp. 260-268.

28. Launay, O., Jean-Claude, N.V., Buu-Hoi, A., Acar, J.F. (1997) Typhoid fever due to a *Salmonella*Typhi strain of reduced susceptibility to fluoroquinolones. ClinMicrobiolInfect.3, pp. 541-544.

29. Parry, C.M., Hien, T.T., Dougan, G., White, N.J., Farrar, J.J. (2002) Typhoid

fever. N Engl J Med. 347, pp. 1770-1782.

30. Rowe, B.,Ward,L.R.,Threlfall, E.J.(1995) Ciprofloxacin resistant *Salmonella* Typhi in UK.Lancet.346, pp.1302.

31. Shah, S.A., Suzuki, H., Hassan, M.R., Saito, R., Safian, N., Idrus, S. (2012) Spatial analysis of environmental factors influencing typhoid endemicity in Kelantan, Malaysia.SainsMalaysiana. 41(7), pp.911-919.

32.Shirakawa, T., Acharya,B.,Kinoshita, S.,Kumagai, S., Gotoh, A., Kawabata, M. (2006) Decreased susceptibility to fluoroquinolones and *gyrA* gene mutation in the *Salmonella enterica*serovarTyphi and Paratyphi A isolated in Katmandu, Nepal in 2003.DiagnMicrobiolInfect Dis.54, pp.299-303.

33.Threlfall, E.J., Ward, L.R., Rowe, B., Raghupathi, S., Chandrasekaran, V., Vandepitte, J., Lemmens, P. (1992) Widespread occurrence of multiple drug-resistant *Salmonella* Typhi in India. Eur J ClinMicrobiol Infect Dis.11, pp.990-993.

34.Threlfall, E.J., Ward, L.R. (2001) Decreased susceptibility to ciprofloxacin in *Salmonella enterica* serotype Typhi, United Kingdom.Emerg Infect Dis. **7**(3), pp. 448-450.

35. Wain, J., Hoa, N.T., Chinh, N.T., Vinh, H., Everett, M.J., et al. (1997) Quinoloneresistant*Salmonella*Typhi in Vietnam: molecular basis of resistance and clinical response to treatment. Clin Infect Dis. 25, pp. 1404-1410.

Legends:

Figure 1: PCR product of QRDR of *gyr*A gene of DR *S*.TyphiKelantan isolates. M: 100 bp DNA marker, -ve: Negative control using DNA of *E.coli* ATCC 25922, lane 1-30: PCR product of 30 DR *S*.TyphiKelantan isolates.

Figure 2: PCR-RFLP pattern of 30 DR*S*.Typhi Kelantan isolates based on digestion at Ser-83 in QRDR of *gyr*A gene with *Tfi*1 restriction enzyme. M: 100 bp DNA marker and -ve: Negative control using DNA of *E.coli* ATCC 25922, NT: Non-treated PCR product and Lane 1-30: PCR products of 30 DR *S*.Typhi Kelantan isolates cut with *Tfi*1 restriction enzyme.

Table 1. Antimicrobial sensitivity and Ser-83 mutation in QRDR of *gyrA*geneof 30DR *S*.Typhi Kelantan isolates.AMP= Ampicillin; CEF=Ceftriaxone; CH=Chloramphenicol; CIP=Ciprofloxacin; NAL=Nalidixic acid; TS=Trimethoprim-sulfamethoxazole; MDR= Multidrug resistant; S= sensitive; R=resistance; Asp= Aspartic Acid; Phe=phenylalanine ;Ser=Serine and – = no mutation.

Table 1

Strain no.	AMP	CEF	NAL	CIP	TS	CH	*Gyr*Agene	
							QRDR 83(TCC[Ser])	QRDR 87(GAC[Asp])
B0188/08	S	S	R	R	S	S	-	-
B7402/05	S	S	R	R	S	S	-	-
B2187/03	S	S	R	R	S	S	-	-
B055B/08	S	S	R	R	S	S	-	-
S0398/03	R	R	S	S	S	S	-	-
B7111/05	S	S	R	R	S	S	-	-
B9107/03	S	S	R	R	S	S	-	-
B7423/05	S	S	R	R	S	S	-	-
B0206/08	S	S	R	R	S	S	-	-
B6236/05	S	S	R	R	S	S	-	-
S1792/05	S	S	R	R	S	S	-	-
B6090/05	S	S	R	R	S	S	-	-
B3952/07	S	S	R	R	S	S	-	-
B0147/08	S	S	R	R	S	S	-	-
S0400/05	S	S	R	R	S	S	-	-
B6786/05	S	S	R	R	S	S	-	-
B8919/03	S	S	R	R	S	S	-	-
S838/03	S	S	R	R	S	S	-	-
B4327/03	S	S	R	R	S	S	-	-
B6743/05	S	S	R	R	S	S	-	-
S0554/05	S	S	R	R	S	S	-	-
FH2172/08	S	S	R	R	S	S	-	-
B10085/02	S	S	R	R	S	S	-	-

B10131/02	S	S	R	R	S	S	-	-
FH00069/09	S	S	R	R	S	S	-	-
S0241/08	S	S	R	R	S	S	-	-
B10085/02	S	S	R	R	S	S	-	-
S0033/09	R	S	R	R	S	S	-	-
S0147/07_MDR	R	S	R	R	R	S	-	-
S00603/06	R	R	S	S	S	S	-	-
B2534/03	S	S	R	S	S	S	-	-
FH00075/08	S	S	R	S	S	S	-	-
FH2164/08	S	S	R	S	S	S	-	-
S00554/05	S	S	R	S	S	S	-	-
S00416/05	S	S	R	S	S	S	-	-
B00095/08	S	S	R	S	S	S	-	-
B7377/05_MDR	R	S	S	S	R	R	**TTC (Phe)**	-

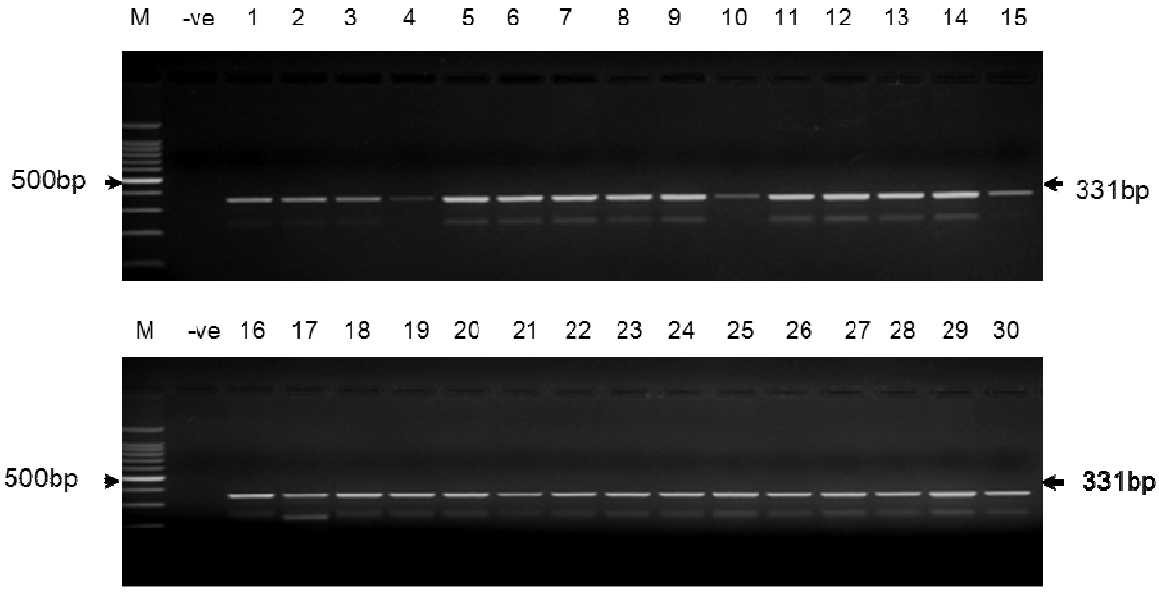

Figure 1

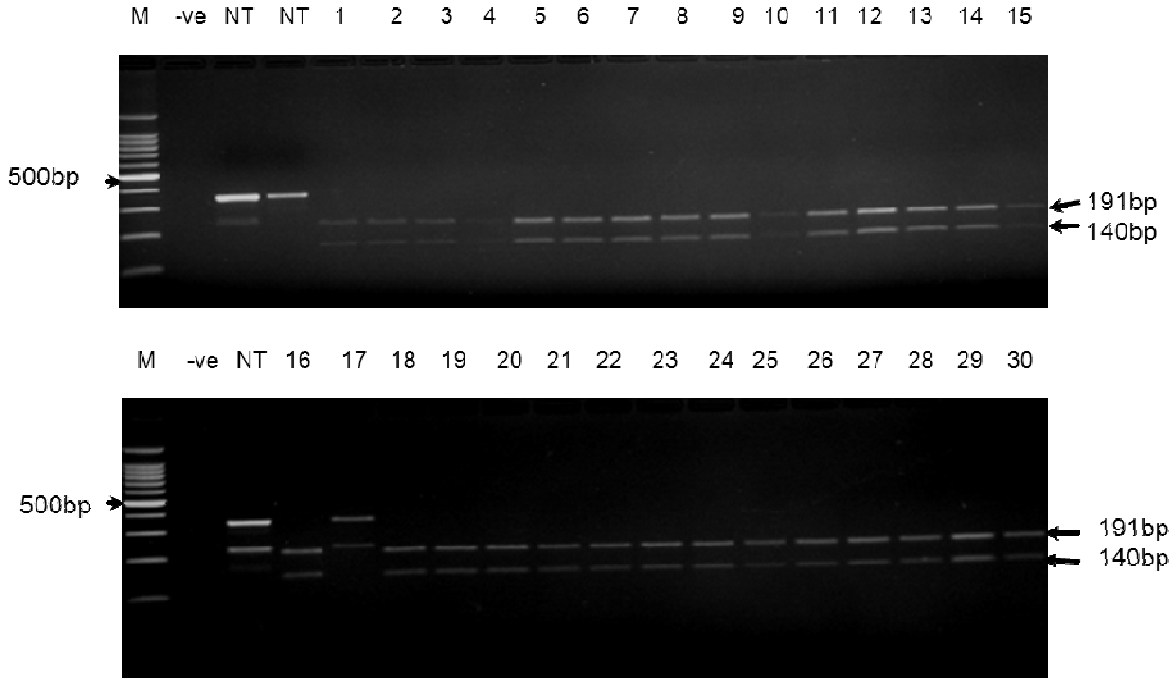
M -ve NT NT 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
500bp
191bp
140bp
M -ve NT 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
500bp
191bp
140bp

Figure 2