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1 Genotyping of *Salmonella* spp. on the basis of CRISPR (Clustered Regularly Interspaced Short
2 Palindromic Repeats).

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13 **ABSTRACT**

14 **Aims:** Bacterial genotyping on the basis of the CRISPR array has been established in *Mycobacterium*
15 *tuberculosis* with a method called spacer oligonucleotide typing (spoligotyping). The spoligotyping
16 method had been widely used for both detection and typing of *M. tuberculosis* complex bacteria.
17 This present study aimed at determining if the CRISPR array in *Salmonella* spp. could be applied to
18 establish a correlationship between serogroup and the fingerprint generated by CRISPR typing.

19 **Methodology and results:** A total of 30 samples were obtained from Diagnostic Veterinary
20 Laboratory, Kota Kinabalu, Sabah. Serogroup was determined on the basis of ELISA (enzyme-linked
21 immunosorbent assay). Four different serogroups were identified which were serogroup B, C, D, and
22 E. DNA (deoxyribonucleic acid) was extracted and PCR (polymerase chain reaction) was performed
23 using primers which were designed to amplify the CRISPR array in *Salmonella* genome. Our results

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24 indicate that there is a correlationship between serogroup obtained using ELISA and the profile
25 generated by CRISPR typing.

26 **Conclusion, significance and impact of study:** CRISPR typing has the potential to be applied for the
27 genotyping of *Salmonella*.

28 ***Keywords***: *Salmonella*, genotyping, serogroup, CRISPR

29 **INTRODUCTION**

30 The first CRISPRs locus were identified over 25 years ago in *Escherichia coli* as ambiguous repeat
31 (Ishino *et al*., 1987) and are known as CRISPR spacer arrays now (Mojica *et al*., 2000; Jansen *et al*.,
32 2002a; 2002b). CRISPR arrays consists of tandem direct repeats (DRs) of 23 to 55 bp (base pair) in
33 length interspaced by equal sized of variable spacer sequences that acquired from bacteriophages or
34 plasmids (Bolotin *et al*., 2005; Mojica *et al*., 2005; Pourcel *et al*., 2005; Boyaval *et al*., 2007). The
35 spacer in CRISPR locus was first applied to subtyping *Mycobacterium tuberculosis* strains and this
36 method was known as spacer-oligonucleotide typing or "spoligotyping" (Groenen *et al*., 1993;
37 Kamerbeek *et al*., 1997). Recently, the "next generation" microbead-spoligotyping method was
38 applied to *Salmonella* in an assay named CRISPOL (for "CRISPR polymorphism")(Fabre *et al*., 2012).
39 Fabre *et al*. (2012) state that there are at least three potential interest of using the polymorphism in
40 *Salmonella* CRISPR locus for clinical microbiology or public health laboratories which are: 1. CRISPR
41 sizing by PCR to compare different isolate of *Salmonella* spp. 2. CRISPOL assay to subtype *Salmonella*
42 serotype Typhimurium or its monophasic variant. 3. Development of PCR assay to target specific
43 *Salmonella* serotype or strain. We aimed to demonstrate that CRISPR based genotyping can be
44 utilized as a diagnostic tool to differentiate different *Salmonella* serogroups prevalent in Sabah.

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47 **MATERIAL AND METHODS**

48 **Sample Collection**

49 *Salmonella* samples (N=30) were obtained from the Diagnostic Veterinary Laboratory, Kota Kinabalu,
50 Sabah. All the *Salmonella* samples were isolated from avian host. The *Salmonella* bacteria were
51 cultured on nutrient agar and MacConkey agar. The MacConkey agar is a selective medium for
52 *Salmonella* which functions to ensure that contaminants are eliminated. The *Salmonella* colony from
53 nutrient agar were transferred to nutrient broth and cultured overnight in incubator shaker at 37°C
54 and 200 rpm (revolutions per minute). The *Salmonella* samples were stored at 25% glycerol stock in
55 -80°C freezer for future use.

56 **Serogrouping**

57 ELISA was carried out as follows. *Salmonella* were cultured overnight at nutrient agar by streak plate
58 method at 37°C. 0.85% saline solution was used for the ELISA test. The ELISA test was done using the
59 *Salmonella* Sero-Quick Group Kit from Statens Serum Institut, Denmark. The protocol for *Salmonella*
60 ELISA test was done according to the manufacturer protocol.

61 **DNA Extraction**

62 DNA was extracted according to modified Kang *et al*. (1998) method. The dry DNA pellet was re-
63 suspended in 100 µl of 1X TE (Tris-EDTA) buffer and stored in -20°C for future use.

64 **PCR of *invA* gene**

65 The primer pair used for this PCR were taken from Cortez *et al*. (2006). PCR was performed in a
66 reaction volume of 25 µl using the GE Healthcare illustra^{TM} puReTaq Ready-To-Go PCR Beads. The
67 preparation of PCR master mix was prepared according to manufacturer protocol. Amplification was
68 carried out in a thermal cycler (MJ Research PTC-200 Peltier Thermal Cycler) using 35 cycles
69 consisting of denaturation for 30 sec at 94°C, annealing for 1 min at 55°C, and extension for 1 min at

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70 72°C, followed by a final extension for 7 min at 72°C. Electrophoresis of amplified products was
71 carried out using 1.0% agarose gel in 1X TBE (Tris/Borate/EDTA) running buffer. The amplified DNA
72 fragments were stained with ethidium bromide and visualized under UV (ultraviolet) light. A 100 bp
73 DNA ladder (New England Biolabs Quick-Load 100 bp DNA Ladder) was used as a reference standard.

74 **Primer Design**

75 Design of CRISPR specific primers was carried out as follows. The complete genome of *Salmonella*
76 *enterica* subsp. *enterica* serovar Typhi strain CT18 was first retrieved from the NCBI (National Centre
77 for Biotechnology Information) with accession number of NC_003198.1. The genome was then
78 analyzed for presence of CRISPR locus using online web tool called CRISPERFinder at http://crispr.u-
79 psud.fr/Server/CRISPRfinder.php. Once the CRISPR locus sequence was identified three pairs of
80 primer set were designs to amplify the CRISPR 1 and CRISPR 2 locus presence in *Salmonella* genome
81 using online web tool called Primer3 at http://bioinfo.ut.ee/primer3-0.4.0/. The primer sequences
82 are listed in Table 1.0.

83 **PCR of CRISPR Locus**

84 PCR was performed in a reaction volume of 20 µl containing 1X Dream Taq. Green Buffer (Thermo
85 Scientific), 0.1 mM dNTPs (deoxynucleoside triphosphate), 2 mM $MgCl_2$ (Magnesium chloride), 5 µM
86 of forward and reverse primer, 1 Unit of Thermo Scientific Dream Taq DNA polymerase and 1 µl of
87 DNA template. Amplification was carried out in a thermal cycler (MJ Research PTC-200 Peltier
88 Thermal Cycler) with initial denaturation of 96°C for 4 min, followed by 35 cycles of 96°C for 30 sec,
89 51°C for 30 sec, 72°C for 1 min and final extension step at 72°C for 2 min. All steps were the same for
90 all primers except for the annealing temperature. The annealing temperature for CRISPR1 primer pair
91 is 48°C, 51°C for CRISPR2 primer pair and 49°C for FARH primer pair. Electrophoresis of amplified
92 products was carried out using 1.0% agarose gel in 1X TBE running buffer. The amplified DNA
93 fragments were stained with ethidium bromide and visualized under UV light. A 100 bp DNA ladder
94 (New England Biolabs Quick-Load 100 bp DNA Ladder) was used as a reference standard.

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95 **RESULTS**

96 *Salmonella* samples (N=30) were test by ELISA for serogrouping. 53% of the samples were from
97 serogroup C, followed by serogroup E with 20%, 17% for serogroup B and 10% for serogroup D. The
98 genus confirmations for all the samples were done by the PCR of *invA* gene (Figure 1.0, 2.0 and 3.0).
99 The expected size for the PCR product is 521 bp. PCR to amplify the CRISPR locus on *Salmonella*
100 genome were done to differentiate the different *Salmonella* serogroups (Figure 4.0, 5.0 and 6.0).

101 **DISCUSSION**

102 The O antigen present in the cell surface of *Salmonella* is extremely polymorphic and is used to
103 determine the bacteria serogroup (Bee & Kwai, 2009). The variation in O antigen structure is due to
104 the different types of sugar present, the arrangement of sugars, the addition of branch sugars and
105 the modifying side groups in which such variation is used to serogroup *Salmonella* isolates (Wyk &
106 Reeves, 1989; Fitzgerald *et al*., 2003; Luk *et al*., 2006). In this study four serogroups were found
107 which were serogroup B, C, D and E. This finding is similar with the finding of Lindberg & Le Minor
108 (1984) and Luk & Lindberg (1991) that stated over than 95% of the *Salmonella* strains that cause
109 infection in human and animal is originate from the serogroup A to E. The identity of the *Salmonella*
110 samples recruited for this study was confirmed by the present of *invA* gene in *Salmonella* genome
111 using PCR method. This was done in order to ensure that all the samples used in this study were from
112 the pure culture of *Salmonella* bacteria. PCR was an effective, rapid, reliable and sensitive method
113 for the detection of *invA* gene present in *Salmonella* genome (Zahraei-Salehi *et al*., 2006). A study
114 from Galán & Curtiss (1989) show that a group of genes (*invA, B, C, D*) confer *Salmonella* the ability to
115 invade cultured epithelial cells. The use of this gene for *Salmonella* identification by PCR method has
116 recently been suggested as this gene were shown to be found in a number of *Salmonella* strain
117 (Galán & Curtiss, 1991). A study by Zahraei-Salehi *et al*. (2006) confirm that the *invA* gene sequence is
118 unique to *Salmonella* and this unique sequence can be used as the PCR target to differentiate
119 *Salmonella* from other organisms. The CRISPR typing was done to differentiate *Salmonella* serogroup

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120 by molecular method and the result was compared with the traditional serogrouping by ELISA
121 method. The amplification profile of *Salmonella* from different serogroups were observed and
122 compared. The principle of this method is that the variation in spacer number that interspaced
123 between the direct repeats will give different lengths of CRISPR array and can be used to rapidly
124 screen *Salmonella* isolates by PCR and gel electrophoresis analysis (Shariat & Dudley, 2014). The
125 CRISPR1 and CRISPR2 primer pairs failed to differentiate some of the samples when compared to the
126 serogrouping result obtained by the ELISA method. Depending on the antibody use for ELISA test,
127 they may lack specificity because of the non-specific agglutination that might happen in some
128 *Salmonella* bacteria as stated by Cheesbrough & Donnelly (1996). Only the CRISPR primer designated
129 as FARH primer was able to completely differentiate all the samples from different serogroups. The
130 reason why CRISPR1 and CRISPR2 primer cannot resolve the serogroup while FARH primer can might
131 be because of the location of the primer along the CRISPR gene. The positions of forward and reverse
132 primer for CRISPR1 along the gene are at nucleotide number 2,925,620 to 2,925,640 and 2,926,778
133 to 2,926,798 respectively. For CRISPR2 the positions of the forward and reverse primer along the
134 gene are at nucleotide number 2,926,215 to 2,926,233 and 2,926,450 to 2,926,470 respectively. For
135 the FARH the positions of forward and reverse primer along the gene are at nucleotide number
136 2,926,294 to 2,926,313 and 2,926,450 to 2,926,470 respectively. It can be seen from the results that
137 some samples from the same serogroup have different amplification profile. This might be because
138 of in one serogroup there were sub-group present as shown in White-Kauffmann-Le-Minor scheme
139 (Grimont & Weill, 2008). Our findings are consistent with those obtained by Fabre *et al*. (2012) and
140 Shariat & Dudley (2014) who used rapid CRISPR size typing to screen *Salmonella* spp. isolates by
141 comparing the amplicon size of PCR product run on agarose gel electrophoresis.
142

143 **CONCLUSION**

144 From this study, it shown that only the FARH primer is able to resolve the samples into their
145 respective serogroup. The serogrouping by ELISA method can be complement with CRISPR typing by

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146 PCR method to get more accurate result as non-specific agglutination might occur using the ELISA
147 method.

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151 Laboratory, Kota Kinabalu, Sabah for providing us the *Salmonella* samples used in this study.

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236 **Attachments (Figures and Table)**

PRIMER NAME	Forward Primer (5'-3')	Reverse Primer (5'-3')	Annealing Temperature (Ta)
CRISPR1	ATT CGT TTT ATC GCC ACC AG	CTG GCA GA T GAG GGA AAT GT	48°C
CRISPR2	AGC AAC CCG TGT CGG ATA	CGC AAC CG G TGT TTT AGT GT	51°C
FARH	GCG TGA AT T GCG GTT TAT C	CGC AAC CG G TGT TTT AGT GT	49°C

237 **Table 1.0** List of primer used for the CRISPR typing.

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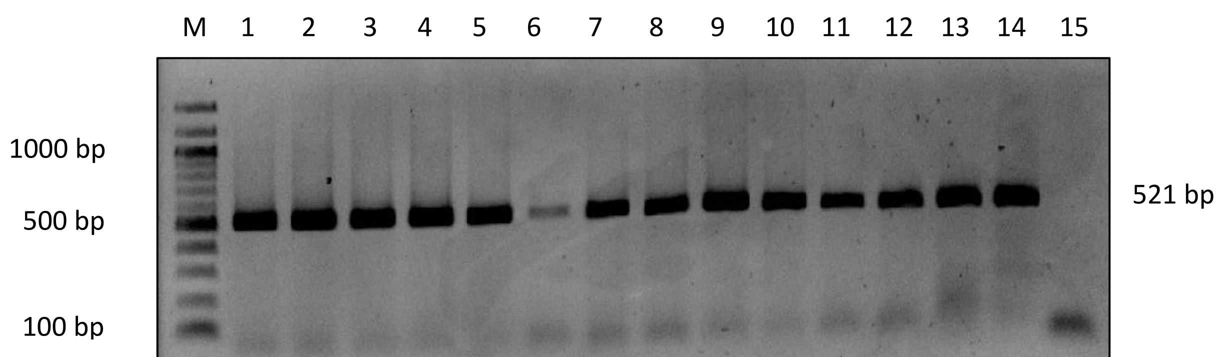

241

242 **Figure 1.0** PCR was done to amplify the *invA* gene on the *Salmonella* bacteria genome for

243 genus confirmation. Lane M: 100 bp marker, lane 1 to 14: The PCR product of *Salmonella*

244 *invA* gene and lane 15: Negative control without DNA template.

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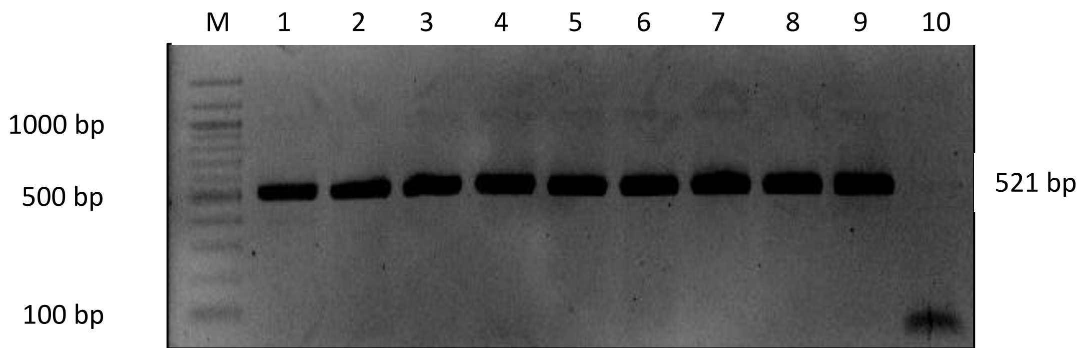
250



251

252 **Figure 2.0** PCR was done to amplify the *invA* gene on the *Salmonella* bacteria
253 genome for genus confirmation. Lane M: 100 bp marker, lane 1 to 9: The PCR
254 product of *Salmonella invA* gene and lane 10: Negative control without DNA
255 template.

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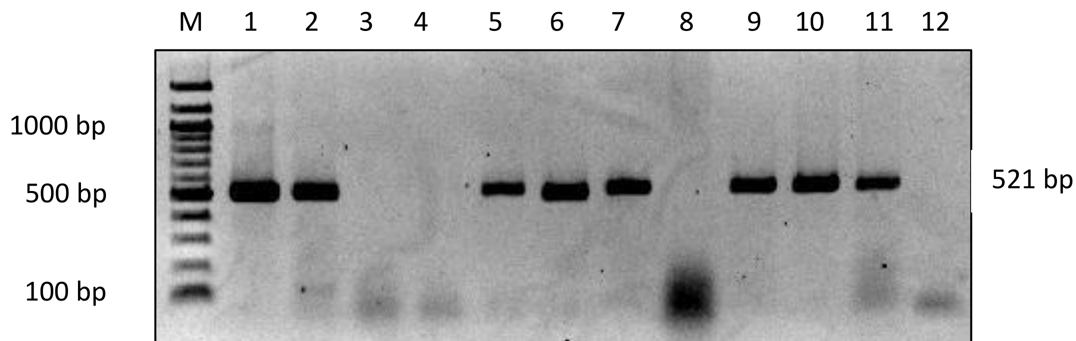

258

259 **Figure 3.0** PCR was done to amplify the *invA* gene on the *Salmonella* bacteria
260 genome for genus confirmation. Lane M: 100 bp marker, lane 1 to 11: The PCR
261 product of *Salmonella invA* gene and lane 12: Negative control without DNA
262 template.

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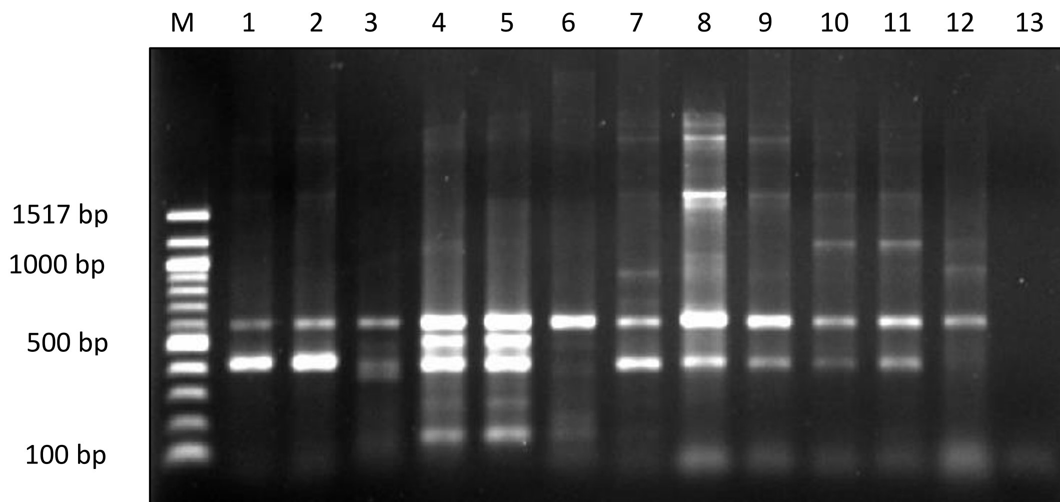

267

268 **Figure 4.0** CRISPR1 primer pair was designed for PCR to amplify the CRISPR locus in *Salmonella*
269 genome for serogrouping differentiation by molecular method. The above agarose gel picture show
270 the amplification profile when *Salmonella* genome was tested with the designed primer. Lane M: 100
271 bp marker, lane1-3: *Salmonella* from serogroup B, lane 4-6: *Salmonella* from serogroup C, lane 7-9:
272 *Salmonella* from serogroup D, lane 10-12: *Salmonella* from serogroup E and lane 13: Negative control
273 without DNA template.

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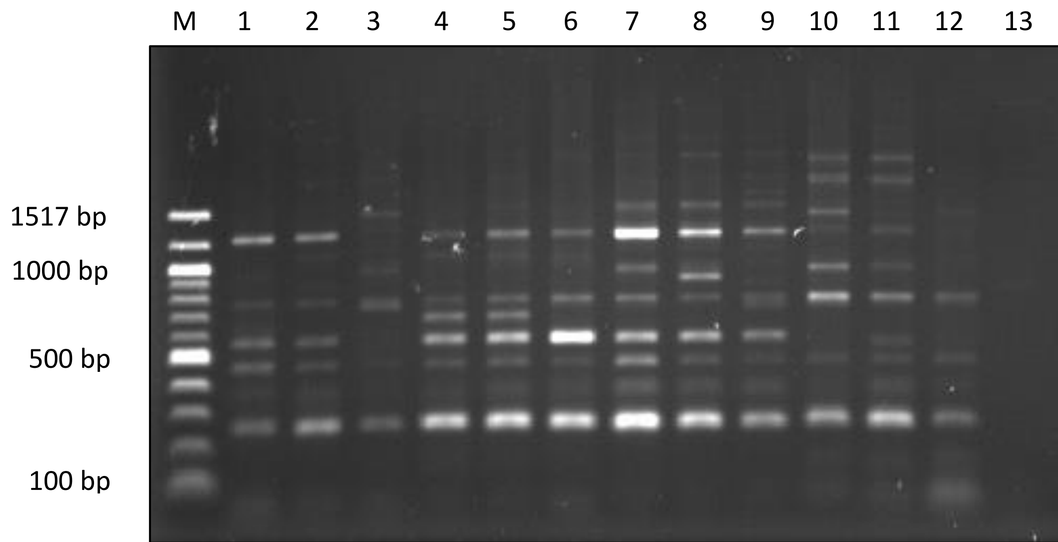

Figure 5.0 CRISPR2 primer pair was designed for PCR to amplify the CRISPR locus in *Salmonella* genome for serogrouping differentiation by molecular method. The above agarose gel picture show the amplification profile when *Salmonella* genome was tested with the designed primer. Lane M: 100 bp marker, lane1-3: *Salmonella* from serogroup B, lane 4-6: *Salmonella* from serogroup C, lane 7-9: *Salmonella* from serogroup D, lane 10-12: *Salmonella* from serogroup E and lane 13: Negative control without DNA template.

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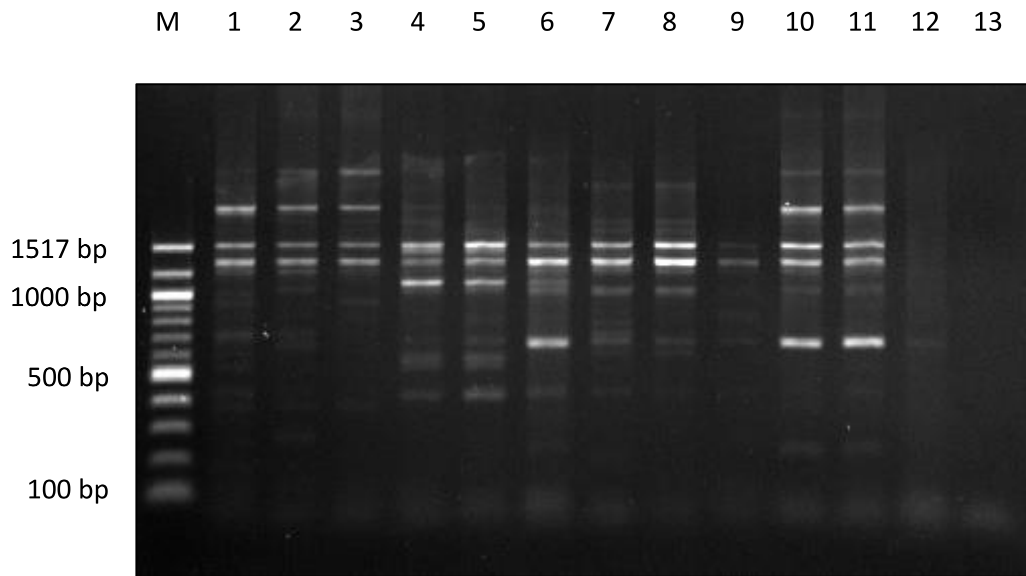

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300 **Figure 6.0** FARH primer pair was designed for PCR to amplify the CRISPR locus in *Salmonella* genome
301 for serogrouping differentiation by molecular method. The above agarose gel picture show the
302 amplification profile when *Salmonella* genome was tested with the designed primer. Lane M: 100 bp
303 marker, lane1-3: *Salmonella* from serogroup B, lane 4-6: *Salmonella* from serogroup C, lane 7-9:
304 *Salmonella* from serogroup D, lane 10-12: *Salmonella* from serogroup E and lane 13: Negative control
305 without DNA template.

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