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

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ABSTRACT

Aims: The CRISPR locus in *Salmonella* genome is comprised of three main components which are the (CRISPR-associated) *cas* genes, an AT-rich leader sequence and the CRISPR array. The length of CRISPR array is determined by the number of spacers within it and varies not only among different organisms but also varies among the bacterial serotypes and strains. This present study aimed at determining if the CRISPR array in *Salmonella* spp. could be applied to establish a correlation between serogroup type and the fingerprint generated by CRISPR typing.
Methodology and results: A total of 30 *Salmonella* samples were obtained from the Veterinary Diagnostic Laboratory, Kota Kinabalu, Sabah. *Salmonella* serogroup was determined using the slide agglutination test. Four different serogroups were identified which were serogroup B, C, D, and E. Deoxyribonucleic acid (DNA) was extracted and polymerase chain reaction (PCR) was performed using primers which were designed to amplify the CRISPR array in *Salmonella* genome. Our results indicate that there is a positive correlation between serogroup results obtained using slide agglutination test and the profile generated by CRISPR typing.
Conclusion, significance and impact of study: CRISPR typing has the potential to be applied for the genotyping of *Salmonella* bacteria.

Keywords: Salmonella, genotyping, serogroup, CRISPR typing

INTRODUCTION

Current traditional serotyping method based on the Kauffmann-White-Le Minor scheme is consider as the gold standard for subtyping *Salmonella* strains (Wattiau *et al*., 2011). However, this method is time consuming and tedious, as well as subjective in interpretation (Lim and Thong, 2009). Such limitation lead to the development of nucleotide-based method for subtyping *Salmonella* isolates. One of the potential genes which could be used for this purpose is the CRISPR gene. The first CRISPR locus were identified over 25 years ago in *Escherichia coli* as ambiguous repeat (Ishino *et al*., 1987) and are known as CRISPR spacer arrays now (Mojica *et al*., 2000; Jansen *et al*., 2002a; Jansen *et al*., 2002b). CRISPR arrays consists of tandem direct repeats (DR) of 23 to 55 bp (base pair) in length interspaced by equal sized of variable spacer sequences that acquired from bacteriophages or plasmids (Bolotin *et al*., 2005; Mojica *et al*., 2005; Pourcel *et al*., 2005; Boyaval *et al*., 2007). The spacer in CRISPR locus was first applied to subtyping *Mycobacterium tuberculosis* strains and this method was known as spacer-oligonucleotide typing or "spoligotyping" (Groenen *et al*., 1993; Kamerbeek *et al*., 1997). The same CRISPR array in *Salmonella* genome could be used for subtyping this bacterium as previous study by Fabre *et al*. (2012) show the polymorphism in this locus are strongly correlated with it serotype. In this study, we aimed to determine if the CRISPR array in the *Salmonella* genome can be utilized to establish a correlation between *Salmonella* serogroup and the fingerprint generated by CRISPR typing.

MATERIALS AND METHODS

Sample collection

Salmonella samples (n=30) were obtained from the Veterinary Diagnostic Laboratory, Kota Kinabalu, Sabah. All the *Salmonella* samples were isolated from dead chicken carcasses (*Gallus gallus domesticus*). The organ from the dead chicken carcasses were taken out and swab using the laboratory swab. The *Salmonella* isolate were then cultured overnight in buffered peptone water for pre-enrichment step. After the pre-enrichment step, *Salmonella* isolates were cultured overnight on Xylose-

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Table 1: Primers used for the CRISPR typing.

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

Figure 1: The arrow above shows the location of the primers along the CRISPR array in the *Salmonella* genome. CRISPR1F: CRISPR1 forward primer, CRISPR2F: CRISPR2 forward primer, FARHF: FARH forward primer, CRISPR1R: CRISPR1 reverse primer, CRISPR2R: CRISPR2 reverse primer and FARHR: FARH reverse primer.

Lysine Deoxycholate (XLD) agar. Several *Salmonella* colonies that grow on the XLD agar were then transferred to bijou bottle containing nutrient agar and kept at 4 °C as the primary stock. All the isolation and purification process of *Salmonella* isolates from the dead chicken carcasses was done by the laboratory technician from the veterinary lab as this study was a collaboration work between Universiti Malaysia Sabah (UMS) and the Veterinary Diagnostic Laboratory, Kota Kinabalu. For long term storage, 25% glycerol stock of the *Salmonella* isolates were prepared from the primary stock and kept at –80 °C freezer for future use. The preparation of the glycerol stock from the primary stock was done as follow. The *Salmonella* isolates from the primary stock was first cultured overnight on the nutrient agar plate. Then several *Salmonella* colonies that grow on the nutrient agar were transferred to nutrient broth and cultured overnight in incubator shaker at 37 °C with 200 rpm (revolutions per minute). The *Salmonella* isolates from the nutrient broth were then stored at 25% glycerol stock in –80°C freezer for future use.

DNA extraction from *Salmonella* isolates

DNA was extracted according to modified Kang *et al*. (1998) method. Prior to DNA extraction step, *Salmonella* isolates from the primary stock was first cultured overnight on nutrient agar. Several colonies that grow on the nutrient agar were then transferred to nutrient broth and cultured overnight in incubator shaker at 37 °C with 200 rpm. The growth *Salmonella* isolates in the nutrient broth was then used for the DNA extraction process. The dry DNA pellet that obtained from the DNA extraction process was re-suspended in 100 µL of 1X TE (Tris-EDTA) buffer and stored in –20 °C for future study.

PCR of *invA* gene

The primer pair used for this PCR were taken from Cortez *et al*. (2006). PCR was performed in a reaction volume of 25 µL using the GE Healthcare illustra^{TM} puReTaq Ready-To-Go PCR Beads. The preparation of PCR master mix was prepared according to manufacturer protocol. Amplification was carried out in a thermal cycler (MJ Research PTC-200 Peltier Thermal Cycler) using 35 cycles consisting of denaturation for 30 sec at 94 °C, annealing for 1 min at 55 °C, and extension for 1 min at 72 °C, followed by a final extension for 7 min at 72 °C. Electrophoresis of amplified products was carried out using 1.0% agarose gel in 1X TBE (Tris/Borate/EDTA) running buffer. The amplified DNA fragments were stained with ethidium bromide and visualized under UV (ultraviolet) light. A 100 bp DNA ladder (New England Biolabs Quick-Load 100 bp DNA Ladder) was used as a reference standard.

Serogrouping of *Salmonella* isolate

Slide agglutination test was carried out to identify the serogroup of all the *Salmonella* isolates. Firstly, *Salmonella* isolates from the primary stock were cultured overnight on the nutrient agar and MacConkey agar by streak plate method and incubated at 37 °C. Since the primary stock used was pure culture of *Salmonella* isolates receive from the veterinary laboratory, the purpose of culturing the *Salmonella* isolates on MacConkey agar was to confirm no contamination occur inside the primary stock. Since *Salmonella* are gram-negative bacteria and also a lactose non-fermenting bacteria, the bacteria colony on the MacConkey agar should appear colorless and transparent. All the *Salmonella* colony cultured on the MacConkey agar appear colorless and transparent indicating no contamination occur inside the primary stock. Therefore, the *Salmonella* colony cultured on the nutrient agar can be proceed for serogrouping using the slide agglutination test. *Salmonella* Sero-Quick Group Kit (SSI Diagnostica, Denmark) is a commercial kit that contain seven type of antisera which can be used to detect *Salmonella* serogroup A, B, C, D, E, F and G. The kit also has Vi

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antiserum to detect capsular antigen on certain *Salmonella* serotype such as *Salmonella* Typhi. Saline solution (0.85%) with pH 7.4 was used as the control. 20 µL of saline solution was apply on the glass slide and mix well with several *Salmonella* colonies from the nutrient agar. If agglutination occur, that particular *Salmonella* isolates are rough strain and therefore untypeable. If no agglutination occur, serogrouping of the *Salmonella* isolates can be proceed following the kit instructions. Firstly, a small drop of antisera (approximately 20 µL) was apply on the glass slide from the dropping bottle. After that, using an inoculating loop several *Salmonella* colonies from the nutrient agar was transferred to the drop of antisera and mix well. The amount of *Salmonella* colonies should be sufficient to give a distinct milky turbidity. Then, the slide was tilt gently and the reaction was observed within 5 to 10 sec timeframe. A positive reaction is seen as a visible agglutination using the naked eye (Figure 4a). A negative reaction was indicated by the persistence of the homogenous milky turbidity and also late or weak agglutination (Figure 4b). The addition of the antisera was added following the order from antisera D, B, C, E, G, F and A. When positive reaction occurs, the serogroup of that *Salmonella* isolate will be the serogroup indicated on the dropping bottle and no further testing is necessary. For example, if the reaction with the antiserum D yielded positive result, then that particular *Salmonella* isolate is belong to serogroup D. No further testing is needed for other antiserum. *Salmonella* isolates that yielded positive result with the antiserum D are further tested with the Vi antiserum. *Salmonella* isolates that are positive with both antiserum D and Vi antiserum are likely to be *Salmonella* serotype Typhi.

Primer design

Design of CRISPR specific primers was carried out as follows. The complete genome of *Salmonella enterica* subspecies *enterica* serotype Typhi strain CT18 was first retrieved from the NCBI (National Centre for Biotechnology Information) with accession number of NC_003198. The genome was then analyzed for the presence of CRISPR locus using online web tool called CRISPERFinder at http://crispr.u-psud.fr/Server/CRISPRfinder.php. Once the CRISPR locus sequence was identified three pairs of primer set were designs to amplify the CRISPR one and CRISPR two locus presence in *Salmonella* genome using online web tool called Primer3 at http://bioinfo.ut.ee/primer3-0.4.0/. The primer sequences are listed in Table 1. and the location of the forward and reverse primer on the *Salmonella* CRISPR array were shown in Figure 1.

PCR of the CRISPR locus

PCR was performed in a reaction volume of 20 µL containing 1X Dream Taq. Green Buffer (Thermo Scientific), 0.1 mM dNTP (deoxyribonucleotide triphosphate), 2 mM $MgCl_2$ (Magnesium chloride), 5 µM of forward and reverse primer, 1 Unit of Thermo Scientific Dream Taq DNA polymerase and 1 µL of DNA template. Amplification was carried out in a thermal cycler (MJ Research PTC-200 Peltier Thermal Cycler) with initial denaturation of 96 °C for 4 min, followed by 35 cycles of 96 °C for 30 sec, 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 all primers except for the annealing temperature. The annealing temperature for CRISPR1 primer pair is 4 8°C, 51 °C for CRISPR2 primer pair and 49 °C for FARH primer pair. Electrophoresis of amplified products was carried out using 1.0% agarose gel in 1X TBE running buffer. The amplified DNA fragments were stained with ethidium bromide and visualized under UV light. A 100 bp DNA ladder (New England Biolabs Quick-Load 100 bp DNA Ladder) was used as a reference standard.

RESULTS

The genus confirmations for all the samples were done by the amplification of *invA* gene in *Salmonella* genome using PCR technique (Figure 2a, 2b and 2c). The expected size for the PCR product is 521 bp. The serogroup of the *Salmonella* samples (n=30) were identified using slide agglutination test. *Salmonella* isolates from serogroup C were the highest with 16 isolates, followed by serogroup E with six isolates, five isolates for serogroup B and three isolates for serogroup D. The serogroup of the *Salmonella* isolates were interpreted based on the agglutination of the bacteria with specific antiserum. PCR to amplify the CRISPR array on *Salmonella* genome were done to differentiate the different *Salmonella* serogroups (Figure 3a, 3b and 3c). The amplification profile on the gel picture were compared with the serogrouping result by slide agglutination test to see if there are any correlation between them. The amplification profile from different *Salmonella* serogroups (B, C, D and E) such as the number of band produce and the size of the PCR products were observed and compared. The result were then interpreted to see if the amplification profile produce by each serogroup were unique to them or not.

DISCUSSION

The identity of the *Salmonella* samples recruited for this study was confirmed by the present of *invA* gene in *Salmonella* genome using PCR method. This was done in order to ensure that all the samples used in this study were from the pure culture of *Salmonella* bacteria. PCR was an effective, rapid, reliable and sensitive method for the detection of *invA* gene present in *Salmonella* genome (Zahraei-Salehi *et al*., 2006). Our results are consistent with the previous study by Cortez *et al*. (2006) who use *invA* gene to detect *Salmonella* bacteria isolated from chicken abattoirs.

The O antigen present in the cell surface of *Salmonella* is extremely polymorphic and was used to determine the bacteria serogroup in the conventional serotyping method (Lim and Thong, 2009). The variation in O antigen structure is due to the different types of

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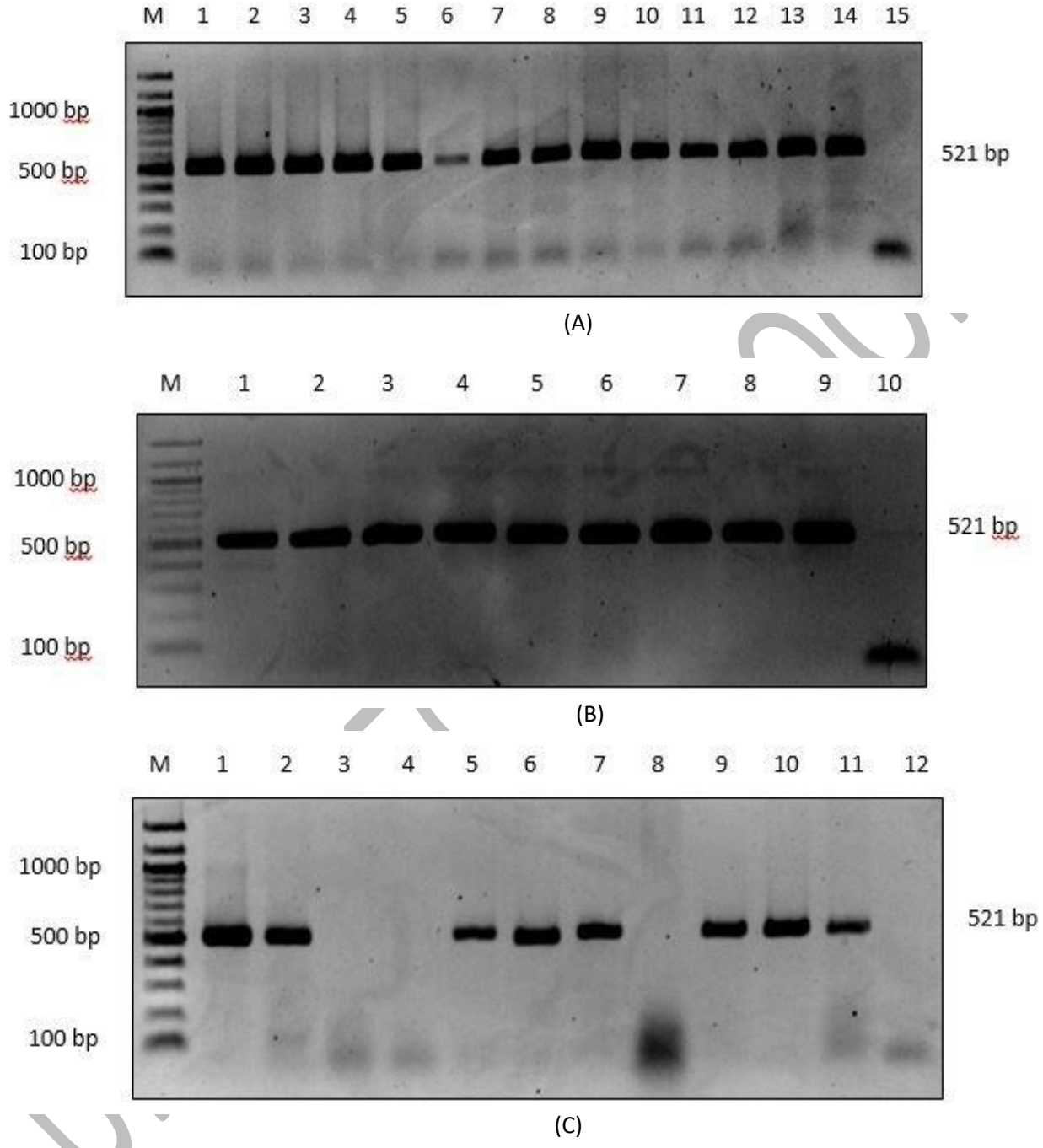

Figure 2.: PCR was done to amplify the *invA* gene on the *Salmonella* bacteria genome for genus confirmation. Lane M: 100 bp marker (a) Lane 1 to 14: The PCR product of *Salmonella invA* gene and lane 15: Negative control without DNA template. (b) Lane 1 to 9: The PCR product of *Salmonella invA* gene and lane 10: Negative control without DNA template. (c) Lane 1 to 11: The PCR product of *Salmonella invA* gene and lane 12: Negative control without DNA template.

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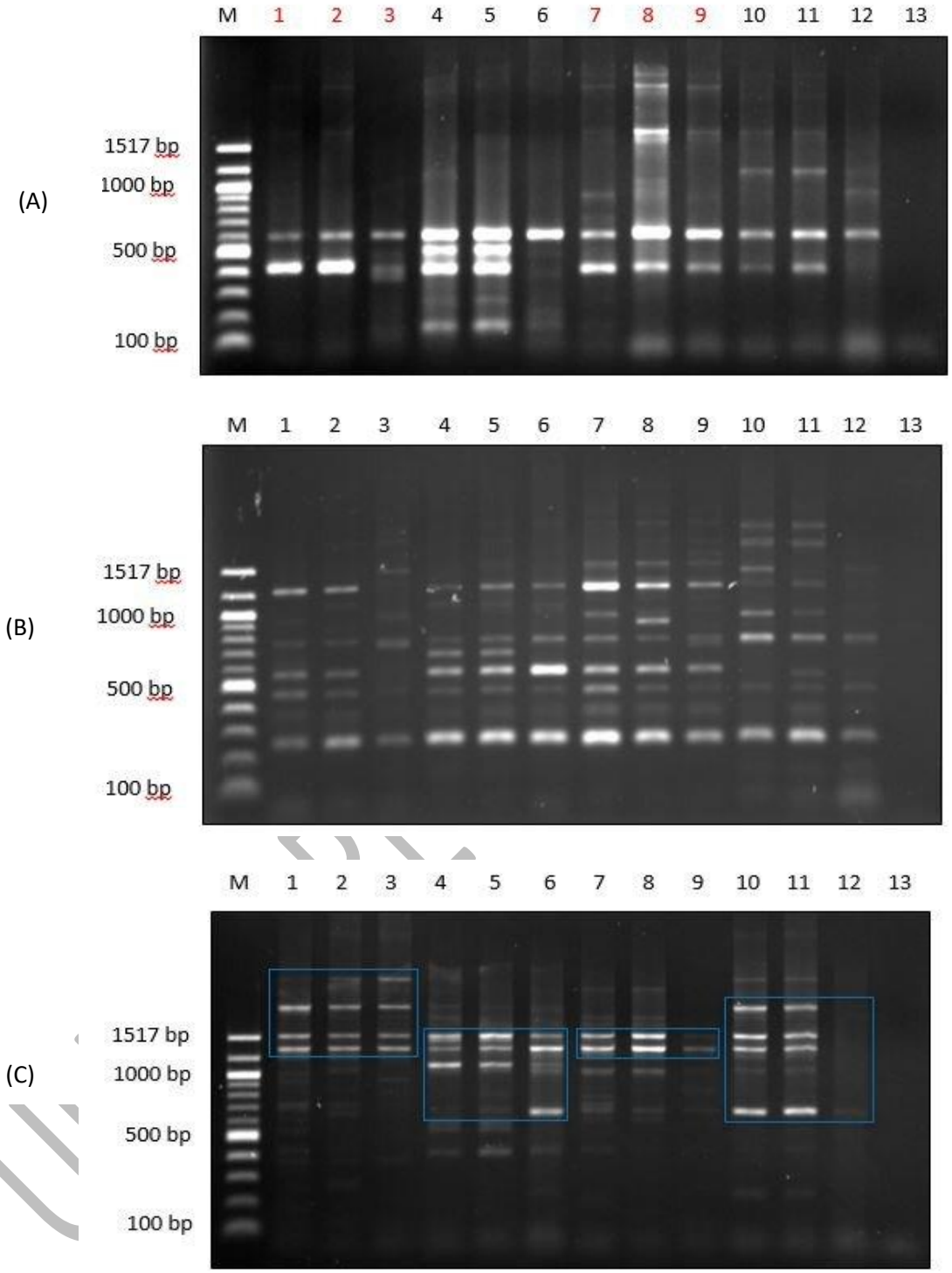

Figure 3: The amplification profile of *Salmonella* genome when tested with the designed primers (a) CRISPR1 primer pair, (b) CRISPR2 primer pair and (c) FARH primer pair. The primers were designed for PCR to amplify the CRISPR locus in *Salmonella* genome for serogrouping differentiation by molecular method. The amplification profile from different serogroups were observed to see if the bands produce were unique for each serogroup type. Lane M: 100 bp marker, lane 1-3: *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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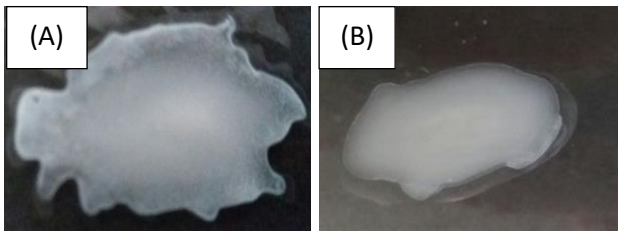

Figure 4: Slide agglutination test using the *Salmonella* Sero-Quick Group Kit (SSI Diagnostica, Denmark) to identify *Salmonella* serogroups, where (a) positive reaction is seen as visible agglutination using the naked eye, while (b) negative reaction is indicated by the persistence of the homogenous milky turbidity.

sugar present, the arrangement of sugars, the addition of branch sugars and the modifying side groups in which such variation is used to serogroup *Salmonella* isolates (Wyk and Reeves, 1989; Fitzgerald *et al*., 2003; Luk *et al*., 2006). In our study four serogroups were found which were serogroups B, C, D and E. This result supports the previous study by Lindberg and Le Minor (1984) and Luk and Lindberg (1991) that stated over than 95% of the *Salmonella* strains that cause infection in human and animal is originate from serogroup A to E where in our study we use *Salmonella* bacteria isolated from dead chicken carcasses.

The CRISPR typing was done to differentiate *Salmonella* serogroup by molecular method and the result was compared with the conventional serogrouping by slide agglutination test. The amplification profile of *Salmonella* from different serogroups were observed and compared for each primer used. As seen from the gel pictures (Figure 3a, 3b and 3c), each primer gives different amplification profile when tested with different serogroup type. This is because different *Salmonella* serogroup have different length of CRISPR array in their genome, therefore their amplification profile are different depending on the number of spacers interspaced between the direct repeat sequences. The amplification profile for each serogroup was affected by the position of the primers along the CRISPR array as shown in figure 1.0. The positions of forward and reverse primer for CRISPR1 along the CRISPR array are at nucleotide number 2,925,620 to 2,925,640 and 2,926,778 to 2,926,798 respectively. For CRISPR2, the positions of the forward and reverse primer along the CRISPR array are at nucleotide number 2,926,215 to 2,926,233 and 2,926,450 to 2,926,470 respectively. For the FARH, the positions of forward and reverse primer along the CRISPR array are at nucleotide number 2,926,294 to 2,926,313 and 2,926,450 to 2,926,470 respectively. As the primers anneal at different location on the CRISPR array during PCR, the amplification profile is different depending on the number of spacer and the direct repeat sequences present between the forward and reverse primers.

When the result of CRISPR typing were compared with the conventional serogrouping result, it shown that only the FARH primer manage to differentiate the serogroup according to the conventional serogrouping result using the slide agglutination test method. The other two primers failed to do so. It can be seen from the gel pictures (Figure 3a, 3b and 3c) that even in the same serogroup different amplification profile was observed. This is due to the presence of sub-group in serogroup C, D and E as reported by Grimont and Weill (2007). This explain why one serogroup could have different amplification profile. When observed carefully on the band in figure 3a, lane 2 (serogroup B) and lane 9 (serogroup D) have same amplification profile. Meanwhile in figure 3b, lane 2 (serogroup B) and lane 6 (serogroup C) also have the same amplification profile. However in figure 3c, all the amplification profile of serogroup B, C, D and E were different. This imply that the CRISPR1 and CRISPR2 primer are not specific enough as the FARH primer and cannot differentiate some serogroups as obtained in slide agglutination test method. The specificity of the designed primer are affected by it location along the CRISPR array as shown in figure 1.0. As for the FARH primer, the amplification profile of different *Salmonella* serogroup were put in blue boxes to show it differences (figure 3c). It can be seen in the figure 3c that serogroup B produced 4 bands, serogroup C (3 bands), serogroup D (2 bands) and serogroup E (4 bands). Although serogroup B and E produce the same number of bands (4 bands), however their PCR product size ranges were different. Our results are consistent with those obtained by Fabre *et al*. (2012) and Shariat and Dudley (2014) who used CRISPR array to subtype *Salmonella* bacteria.

CONCLUSION

From this study, it shown that there are correlation between the polymorphism in *Salmonella* CRISPR array with it serogroup type. The CRISPR array in *Salmonella* genome could become a new molecular subtyping tool for *Salmonella* bacteria.

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