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RESEARCH ARTICLE

Genetic Diversity in *Salmonella* Isolates from Ducks and their Environments in Penang, Malaysia using Enterobacterial Repetitive Intergenic Consensus

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ABSTRACT

A total of 107 *Salmonella* isolates (37 *S. typhimurium*, 26 *S. hadar*, 15 *S. enteritidis*, 15 *S. braenderup*, and 14 *S. albany*) isolated from ducks and their environments in Penang, Malaysia were typed using enterobacterial repetitive intergenic consensus (ERIC) to determine their genetic diversity. Analysis of the *Salmonella* strains by ERIC produced DNA fingerprints of different sizes for differentiation purposes. The DNA fingerprints or band sizes ranged from 14-8300bp for *S. Typhimurium*, 146-6593bp for *S. hadar*, 15-4929bp for *S. enteritidis*, 14-5142bp for *S. braenderup* and 7-5712bp for *S. albany*. Cluster analysis at a coefficient of 0.85 grouped the *Salmonella* strains into various clusters and singletons. *S. typhimurium* were grouped into 10 clusters and 6 singletons, *S. Hadar* were grouped into 3 clusters and 18 singletons, *S. enteritidis* were grouped into 3 clusters and 7 singletons, *S. braenderup* were grouped into 4 clusters and 7 singletons, and *S. albany* were grouped into 3 clusters and 7 singletons with discriminatory index (*D*) ranging from 0.92-0.98. ERIC proved to be a useful typing tool for determining the genetic diversity of the duck *Salmonella* strains.

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INTRODUCTION

Salmonella spp. together with *Citrobacter*, *Enterobacter*, *Escherichia coli*, *Klebsiella*, *Proteus*, *Shigella* and *Yersinia* spp. are members of the *Enterobacteriaceae* family (Carattoli, 2009; Adzitey, 2011). These members are differentiated from each other by their morphological, biochemical and their genetic make-up. While the morphological and biochemical characteristics are very useful in distinguishing bacteria species, the use of molecular techniques based on the genetic constituent of bacteria provide better and more robust means of differentiating bacteria species, and even below species level. Molecular techniques have found their way in recent times in diverse applications. They are used in DNA cloning, detection and diagnosis of infectious and hereditary diseases, identification of genetic fingerprints, detection and confirmation of bacteria and parasites, and so on (Babar *et al*., 2012; Adzitey *et al.*, 2013; Shahzad *et al.*, 2012; Wai *et al*., 2012).

Important foodborne pathogens like *Salmonellae* have widely been studied using molecular techniques such as pulsed field gel electrophoresis (PFGE), random amplified polymorphic deoxyribonucleic acid (RAPD), enterobacterial repetitive intergenic consensus (ERIC), repetitive extragenic palindromic (REP), etc to determine their genetic diversity and/or for outbreak investigations (Weigel *et al*., 2004; Lim *et al*., 2005; Shabarinath *et al.*, 2007). *Salmonellae* such as *S. enteritidis*, *S. typhimurium* and *S.* Stanley in recent times have been responsible for some foodborne disease outbreaks (Suresh *et al*., 2006; Anonymous, 2012a). Furthermore, contact with ducklings or the consumption of duck eggs, meats or products have been associated with *Salmonella* infection or death of affected persons (Merritt and Herlihy, 2003; Noble *et al.*, 2012). Ducks, their rearing and processing environments have also been found to be important sources of *Salmonellae* (Pan *et al.*, 2010; Adzitey *et al.*, 2011; Adzitey *et al.*, 2012a; Adzitey *et al.*, 2012b; Anonymous, 2012b).

Because of the involvement of *Salmonellae* strains in foodborne outbreaks and their occurrence in ducks and their environmental sources, the use of molecular techniques to accurately type them is important for outbreak investigations, for determining their genetic diversities etc. The significance of ducks in the provision of meat, eggs, etc to humans cannot be overemphasized (Adzitey *et al.,* 2012c). Duck production is important in Malaysia and forms an integral part of Malaysians agriculture economy. Malaysia also makes significant contribution to the total duck meat consumed worldwide (Adzitey *et al*., 2012a). Therefore this study was conducted to determine the genetic diversity of *Salmonella* strains isolated from ducks and their environmental sources in Penang, Malaysia.

MATERIALS AND METHODS

***Salmonella* strains:** One hundred and seven *Salmonella* serovar cultures previously isolated from ducks and their environmental sources in Penang, Malaysia between 2009 and 2010 were used for this study (Adzitey *et al*., 2012a). The culture isolates were obtained from the specimen samples including feces (n=36), intestines (n=25), cloaca (n=14), soil (n=11), washing water (n=8), pond water (n=3), carcass rinse (n=2), drinking water (n=2), transporting crate (n=2), floor (n=2), table surface (n=1) and feed (n=1). The cultures serovar belongs to 37 *S. typhimurium*, 26 *S. hadar*, 15 *S. enteritidis*, 15 *S. braenderup* and 14 *S. albany*.

DNA Extraction: A 10ml of Trypticase-Soy Broth (Merck, Germany) was inoculated with a colony of pure *Salmonella* and incubated at 37°C overnight. One ml of the overnight culture was centrifuged for 2min at 14,000xg. Pelleted bacterial cells were subjected to DNA extraction using Wizard® Genomic DNA Purification Kit by following the manufacturer's instructions.

ERIC analysis of duck *Salmonella* isolates: Extracted DNA was typed using modified ERIC PCR reaction and the (22-mer) primer ERIC-1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') described by Versalovic *et al.* (1991) and Weigel *et al.* (2004). ERIC PCR was carried out in a 25µl volume containing 12.5µl mastermix (Promega, USA), 7µl nuclease free water, 2µl 25mM $MgCl_2$, 2.5µl template DNA and 0.5µl of each primer (2µM concentration). Amplification was done with the following temperature cycle: 1 cycle at 95°C for 2min; followed by 35 cycles at 90°C for 30s, 52°C for 1min and 65°C for 8min; and 1 cycle at 65°C for 16min.

All amplifications were performed using thermal cycler system (Biometra®, Germany). Amplicons (2µl) were stained with EZ-Vision® One DNA Dye (10µl), loaded on a 1.5% agarose gel and electrophorezed at 90V for 1h 30min. VC 1kb and VC 100bp DNA ladders (Vivantis) were used as the molecular weight marker and the amplicons were visualized under UV transilluminator gel imaging system (Bio-Rad Gel Imaging System).

Cluster analysis and calculation of discriminatory index: Cluster analysis and calculation of discriminatory index was done individually for the various *Salmonella* serovars. DNA band sizes were determined using the detect band button of the NTSYSpc Version 2.2. Band sizes were then scored as presence of DNA band (a score '1') and absence of DNA band (a score '0'). These scores were entered in NTedit to obtain a data matrix and then inserted in NTSYSpc Version 2.2 computer software for the construction of dendrogram based on simple matching coefficient and UPGMA (Un-weighed Pair-Group Arithmetic Average Clustering) cluster analysis to determine the genetic relatedness of the *Salmonella* strains. Clustering was defined at a coefficient of 0.85 and *Salmonella* strains not belonging to any particular cluster were referred to as singleton (independent strain). The discriminatory index (*D* value) was calculated according to Hunter and Gaston (1988).

RESULTS AND DISCUSSION

In this study, ERIC was used to analyze 107 *Salmonellae* isolated from ducks and their environment in Penang, Malaysia between 2009 to 2010. ERIC analysis of the *Salmonella* strains produced DNA fingerprints of different sizes for differentiation purposes. The DNA fingerprints or band sizes ranged from 14-8300bp for *S. typhimurium*, 146-6593bp for *S. hadar*, 15-4929bp for *S. enteritidis*, 14-5142bp for *S. braenderup* and 7-5712bp for *S. albany*. DNA fingerprints were analyzed using NTSYSpc Version 2.2, and separate dendrograms were constructed for *S. Typhimurium, S. Hadar, S. Enteritidis, S. Braenderup*, and *S. albany*. Fig. (1a) is a representative agarose gel showing ERIC profiles of selected isolates of *Salmonella*. Dendrogram showing the genetic relatedness of *S. typhimurium* isolated from ducks and their environmental sources performed by ERIC is shown in Fig. (1b). Table 1 and 2 showed the ERIC clusters and singletons for the various *Salmonella* strains.

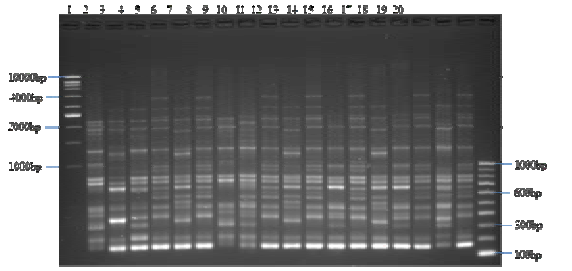

Fig. 1a: ERIC profiles of selected isolates of *Salmonella*. Lane 1: kb DNAa Ladder, Vivantis: lanes 2-19: Salmonella strains isolated from ducks and their environmental samples; lane 20: 100bp DNA Ladder, Vivantis

ERIC analysis and clustering of the *Salmonella* strains at a coefficient of 0.85 produced 10 clusters and 6 singletons for *S. typhimurium* at a *D* value of 0.94, 3 clusters and 18 singletons for *S. hadar* at a *D* value of 0.98, 3 clusters and 7 singletons for *S. enteritidis* at a *D* value of 0.92, 4 clusters and 7 singletons for *S. braenderup* at a *D* value of 0.96, and 3 clusters and 7 singletons for *S. albany* at a *D* value of 0.95. Clusters consisted of 2 or more *Salmonella* strains, examples of which were *S. typhimurium* cluster 1 (TEric1), *S. hadar*

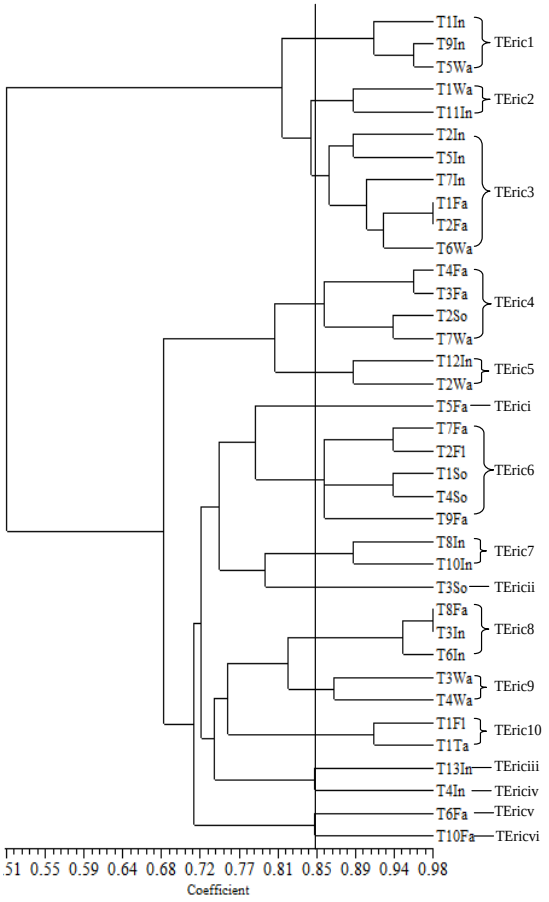

Fig. 1b: Dendogram showing the genetic relatedness of S. *typhimurium* isolated from ducks and their environmental sources performed by ERIC-PCR. TEricl-TEric10=S. *typhimurium* cluster 1-10 by ERIC; TEici-TRapdvi=S. *typhimurium* singleton i-vi by ERIC; T=S.*Typhimurium*; 1-13=strain number; In=intestines; Fa=Feces; Wa=wash water; So=soil; F1=floor swab and Ta=table swab.

Table 1: Clusters and Singletons of *S. typhimurium* and *S. hadar* by ERIC analysis

S. Typhimurium			*S. Hadar*		
Source	Code	Clusters/ singletons	Source	Code	Clusters/ singletons
Intestines	T1In	TEric1	Feces	H7Fa	HEric1
Intestines	T9In	TEric1	Cloacal swab	H6Cl	HEric1
Wash water	T5Wa	TEric1	Feces	H3Fa	HEric2
Intestines	T11In	TEric2	Soil	H2So	HEric2
Wash water	T1Wa	TEric2	Soil	H3So	HEric2
Intestines	T2In	TEric3	Pond water	H2Po	HEric2
Intestines	T5In	TEric3	Feces	H4Fa	HEric3
Intestines	T7In	TEric3	Cloacal swab	H4Cl	HEric3
Wash water	T6Wa	TEric3	Cloacal swab	H9Cl	HErici
Feces	T1Fa	TEric3	Cloacal swab	H8Cl	HEricii
Feces	T2Fa	TEric3	Cloacal swab	H10Cl	HEriciii
Wash water	T7Wa	TEric4	Pond water	H3Po	HEriciv
Feces	T3Fa	TEric4	Feces	H8Fa	HEricv
Feces	T4Fa	TEric4	Cloacal swab	H7Cl	HEricvi
Soil	T2So	TEric4	Feces	H6Fa	HEricvii
Intestines	T12In	TEric5	Cloacal swab	H1Cl	HEricviii
Wash water	T2Wa	TEric5	Feces	H1Fa	HEricix
Feces	T7Fa	TEric6	Cloacal swab	H2Cl	HEricx
Feces	T9Fa	TEric6	Soil	H4So	HEricxi
Soil	T1So	TEric6	Drinking water	H1Dr	HEricxii
Soil	T4So	TEric6	Soil	H1So	HEricxiii
Floor swab	T2Fl	TEric6	Pond water	H1Po	HEricxiv
Intestines	T8In	TEric7	Feces	H2Fa	HEricxv
Intestines	T10In	TEric7	Feces	H5Fa	HEricxvi
Intestines	T3In	TEric8	Cloacal swab	H5Cl	HEricxvii
Intestines	T6In	TEric8	Cloacal swab	H3Cl	HEricxviii
Feces	T8Fa	TEric8			
Wash water	T3Wa	TEric9			
Wash water	T4Wa	TEric9			
Table swab	T1Ta	TEric10			
Floor swab	T1Fl	TEric10			
Feces	T5Fa	TErici			
Soil	T3So	TEricii			
Intestines	T13In	TEriciii			
Intestines	T4In	TEriciv			
Feces	T6Fa	TEricv			
Feces	T10Fa	TEricvi			

TEric1-TEric10 = *S. typhimurium* clusters; TErici-TEricvi = S. *Typhimurium* singletons; HEric1 to HEric3 = *S. hadar* clusters; HErici-HEricxviii = *S. hadar* singletons

cluster 1 (HEric1), *S. enteritidis* cluster 1 (EEric1), *S. braenderup* cluster 1 (BEric1), *S. albany* cluster 1 (AEric1) and many more (Table 1 and 2). *Salmonella* strains in the same cluster are genetically more related. *Salmonella* strains in the same cluster but isolated from different sources could suggest possible cross contamination. An example is *S. typhimurium* cluster 1 (TEric1) which consists of two isolates from intestines and one isolate from wash water (Table 1). *S. typhimurium* from the wash water sample might have been contaminated by intestinal contents since duck intestines rather than wash water samples are potential primary reservoirs for *Salmonella* spp. Singletons (single isolates) also occurred in all groups of *Salmonella* serovars (Table 1 and 2), e.g. *S. typhimurium* T5Fa (TErici), *S. hadar* H9Cl (HErici), *S. enteritidis* E7Fa (EErici), *S. braenderup* B1So (BErici) etc. *Salmonella* strains belonging to these groups were genetically more diverse from other *Salmonella* strains which were recognized as singletons.

ERIC depends on repetitive DNA elements within *Salmonella* strains which were amplified during PCR process to produce DNA fingerprints of different sizes. This technique has been used by other workers to successfully determine the genetic diversity of *Salmonella* spp. for epidemiological studies. Weigel *et al.* (2004) used Rep-PCR which included ERIC primer to detect genetic differences among 68 *Salmonella* isolates and concluded that Rep-PCR would be the preferred method for transmission studies of *Salmonella* especially in cases where precision is necessary because of its high reproducibility and its ability to differentiate closely related isolates.

ERIC PCR was able to differentiate *S.* Essen, *S.* Hillington and *S.* Ruanda which seem to be genetically related by RAPD and SSCP profiles (Lim *et al.*, 2005). Shabarinath *et al.* (2007) genotyped 12 isolates of *S.* Weltevreden by ERIC and RAPD PCR's and suggested that, genetically diverse *S.* Weltevreden strains were prevalent in the sea foods they examined.

Hunter and Gaston (1988) reported that a discriminatory index (*D* value) >0.90 is desirable and the typing results can be interpreted with confidence. This study showed that the discriminatory index of all the *Salmonella* strains at a coefficient of 0.85 was >0.90, thus the ERIC PCR was a valuable typing tool for determining the genetic diversity of the duck *Salmonella* strains. ERIC successfully typed all the *Salmonella* strains and indicated that they were genetically diverse. Furthermore, the distribution of *Salmonella* strains isolated from the same

Table 2: Clusters and Singletons of *S. albany*, *S. braenderup* and *S. enteritidis* by ERIC analysis

S. albany			*S. braenderup*			*S. enteritidis*		
Source	Code	Clusters/singletons	Source	Code	Clusters/singletons	Source	Code	Clusters/singletons
Carcass rinse	A1Ca	AEric1	Cloacal swab	B1Cl	BEric1	Intestines	E4In	EEric1
Carcass rinse	A3Ca	AEric1	Cloacal swab	B3Cl	BEric1	Intestines	E5In	EEric1
Intestines	A1In	AEric2	Feces	B4Fa	BEric2	Intestines	E1In	EEric2
Carcass rinse	A2Ca	AEric2	Cloacal swab	B4Cl	BEric2	Feces	E6Fa	EEric2
Feces	A2Fa	AEric3	Intestines	B2In	BEric3	Feces	E8Fa	EEric2
Feces	A3Fa	AEric3	Feces	B1Fa	BEric3	Feces	E9Fa	EEric2
Soil	A1So	AEric3	Intestines	B1In	BEric4	Intestines	E2In	EEric3
Feces	A1Fa	AErici	Intestines	B3In	BEric4	Intestines	E3In	EEric3
Feces	A4Fa	AEricii	Soil	B1So	BErici	Feces	E7Fa	EErici
Soil	A2So	AEriciii	Feces	B3Fa	BEricii	Intestines	E6In	EEricii
Crate swab	A1Cr	AEriciv	Cloacal swab	B2Cl	BEriciii	Feces	E2Fa	EEriciii
Crate swab	A2Cr	AEricv	Intestines	B4In	BEriciv	Feces	E5Fa	EEriciv
Feed	A1Fe	AEricvi	Intestines	B5In	BEricv	Feces	E3Fa	EEricv
Feces	A5Fa	AEricvii	Feces	B2Fa	BEricvi	Feces	E4Fa	EEricvi
			Wash water	B1Wa	BEricvii	Feces	E1Fa	EEricvii

AEric1-AEric3 = *S. albany* clusters; BEric1-BEric4 = *S. braenderup* clusters; EEric1-EEric3 = *S. enteritidis* clusters; AErici-AEricvii = *S. albany* singletons; BErici-ABricvii = *S. braenderup* singletons; EErici-EEricxvii = *S. enteritidis* singletons

or different place(s) in the same cluster(s) possibly suggest that *Salmonella* strains sharing similar genotypes were circulating within ducks, their rearing and processing environments in Penang, Malaysia between 2009 and 2010. ERIC also provided a cheaper, easier and rapid means of typing the duck *Salmonella* strains. Thus ERIC can be used together with PFGE to study the genetic diversity or outbreak investigations of *Salmonella* spp. This is because PFGE is generally known to have better discriminatory power and/or reproducibility, which can sometimes be lacking in ERIC (Wassenaar and Newell, 2000; Adzitey *et al*., 2013). Furthermore, the use of two or more primers, typing methods or comparison of duck *Salmonella* isolates to that of different animal species is recommended for further studies.

Conclusion: To our knowledge, this is the first extensive report on the use of ERIC PCR to determine the genetic diversity of *Salmonella* spp. isolated from ducks and their environment in Malaysia. ERIC analysis at a coefficient of 0.85 grouped the 107 *Salmonella* strains into various clusters and singletons, and indicated that they were genetically diverse. The ERIC PCR adapted was a useful tool for determining the genetic diversity of the *Salmonella* strains isolated from ducks and their environmental samples.

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REFERENCES

Adzitey F, N Huda and GRR Ali, 2013. Molecular techniques for detecting and typing of bacteria, advantages and application to foodborne pathogens isolated from ducks, 3 Biotech, 3: 97-107.

Adzitey F, G Rusul and N Huda, 2012a. Prevalence and antibiotic resistance of *Salmonella* serovars in ducks, duck rearing and processing environments in Penang, Malaysia. Food Res Int, 45: 947-952.

Adzitey F, N Huda and GRR Ali, 2012b. Prevalence and antibiotic resistance of *Campylobacter*, *Salmonella* and *L. monocytogenes* in ducks: a review. Foodborne Pathog Dis, 6: 498-505.

Adzitey F, GRR Ali, N Huda, T Cogan and J Corry, 2012c. Prevalence, antibiotic resistance and RAPD typing of *Campylobacter* species isolated from ducks, duck rearing and processing environments in Penang, Malaysia. Int J Food Microbiol, 154: 197-205.

Adzitey F, 2011. *Escherichia coli*, it prevalence and antibiotic resistant in Malaysia-a mini review. Microbiol J, 1: 47-53.

Adzitey F, N Huda and R Gulam, 2011. Comparison of media for the isolation of *Salmonella* (XLD and Rambach) and *Listeria* species (ALOA and Palcam) in naturally contaminated duck samples. Int J Food Safety, 13: 20-25.

Babar ME, A Nadeem, T Hussain, A Wajid, SA Shah, A Iqbal, Z Sarfraz and M Akram, 2012. Microsatellite marker based genetic diversity among four varieties of Pakistani Aseel Chicken. Pak Vet J, 32: 237-241.

Carattoli A, 2009. Resistance plasmid families in *Enterobacteriaceae.* Antimicrob Agents Chemother, 53: 2227-2238.

Anonymous, 2012a. Multi-country outbreak of *Salmonella* Stanley infections update. EFSA J, 10: 2893.

Anonymous, 2012b. Scientific report of EFSA and ECDC the European Union summary report on trends and sources of zoonoses, zoonotic agents and foodborne outbreaks in 2010. EFSA J, 10: 2597.

Hunter PR and MA Gaston, 1988. Numerical index for the discriminatory ability of typing systems. An application of Simpson's index of diversity. J Clin Microbiol, 26: 2465-2466.

Lim H, KH Lee, CH Hong, GJ Bahk and WS Choi, 2005. Comparison of four molecular typing methods for the differentiation of *Salmonella* spp. Int J Food Microbiol, 5: 411-418.

Merritt TD and C Herlihy, 2003. *Salmonella* outbreak associated with chicks and ducklings at childcare centres. Med J Aust, 179: 63-64.

Noble DJ, C Lane, CL Little, R Davies, E De Pinna, L Larkin and D Morgan, 2012. Revival of an old problem: an increase in *Salmonella enterica* serovar *Typhimurium* definitive phage type 8 infections in 2010 in England and Northern Ireland linked to duck eggs. Epidemiol Infect, 140: 146-149.

Pan ZM, SZ Geng, YQ Zhou, ZY Liu, Q Fang, BB Liu and XA Jiao, 2010. Prevalence and antimicrobial resistance of *Salmonella* sp. isolated from domestic animals in Eastern China. J Anim Vet Adv, 9: 2290-2294.

Shabarinath S, HS Kumar, R Khushiramani, I Karunasagar and I Karunasagar, 2007. Detection and characterization of *Salmonella* associated with tropical seafood. Int J Food Microbiol, 114: 227-233.

Shahzad W, K Mehmood, R Munir, W Aslam, M Ijaz, R Ahmad, MS Khan and AJ Sabir, 2012. Prevalence and molecular diagnosis of *Fasciola hepatica* in sheep and goats in different districts of Punjab, Pakistan. Pak Vet J, 32: 535-538.

Suresh T, AA Hatha, D Sreenivasan, N Sangeetha and P Lashmanaperumalsamy, 2006. Prevalence and antimicrobial resistance of *Salmonella Enteritidis* and other Salmonellas in the eggs and egg-storing trays from retail markets of Coimbatore, South India. Food Microbiol, 23: 294-299.

Versalovic J, T Koeuthand and JR Lupski, 1991. Distribution of repetitive DNA sequences in *Eubacteria* and application to fingerprinting of bacterial genomes. Nucleic Acids Res, 19: 6823-6831.

Wai SS, AA Saleha, Z Zunita, L Hassan and A Jalila, 2012. Occurrence of co-infection of *Helicobacter pullorum* and *Campylobacter* spp. in broiler and village (indigenous) chickens. Pak Vet J, 32: 503-506.

Wassenaar TM and DG Newell 2000. Genotyping of *Campylobacter* spp. Appl Environ Microbiol, 66: 1-9.

Weigel RM, B Qiao, B Teferedegne, DK Suh, DA Barber, RE Isaacson and BA White, 2004. Comparison of pulsed field gel electrophoresis and repetitive sequence polymerase chain reaction as genotyping methods for detection of genetic diversity and inferring transmission of *Salmonella*. Vet Microbiol, 100: 205-217.