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RESEARCH ARTICLE

Molecular Characterization of Non-typhoidal *Salmonella* (NTS) Isolated from Salad Vegetables in Terengganu, Malaysia

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Abstract Salad vegetables are the important sources of vitamins and minerals for human diet. However, salad vegetables are potential vehicles for non-typhoidal *Salmonella* spp. (NTS) as reported in our previous study. Such contamination posed significant food poisoning risk to consumer. The aims of present study include determination of the virulence genes prevalence in NTS isolates in samples from retail markets and farms. Besides, characterization of NTS isolates was performed to determine their genetic relatedness. Multiplex PCR assay was done to detect the presence of 15 virulence genes in 58 NTS isolates. The genetic relatedness of 33 NTS belonging to *S*. Weltevreden, *S*. Albany, *S*. Hvittingfoss, *S*. Aberdeen, *S*. Poona and *S*. Corvallis were characterized using ERIC-PCR and BOX-PCR typing methods. Results showed the presence of 13 virulence genes (*spiA*, *pagC*, *msgA*, *invA*, *sipB*, *prgH*, *spaN*, *orgA*, *tolC*, *sitC*, *lpfC*, *sifA* and *sopB*) among all the isolates with 53 (91.38%) isolates carried more than 11 virulence genes, while other five isolates carried the genes in the range of six to nine types. None of the isolates possessed *spvB* and *pefA* genes. Major clusters were observed at a genetic distance percentage of 90% for both ERIC- and BOX-PCR with discriminatory index of 0.917 and 0.873, respectively. In terms of source and type of sample, all NTS isolates were found diverse without clear association, thus no specific cluster was found. In summary, this study demonstrated the potential risks of NTS from salad vegetables to cause foodborne diseases and they were commonly found to be related with regard to their specific serovars.

Keywords: Salad vegetables, non-typhoidal *Salmonella*, virulence genes, genetic relatedness, ERIC-PCR, BOX-PCR.

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Introduction

Salad vegetables are widely consumed because they are nutritious and rich in minerals. However, salad vegetables were also found contaminated with non-typhoidal *Salmonella* (NTS) [3]. NTS belong to *Salmonella enterica* subspecies *enterica* and it is one of the leading infectious foodborne pathogens causing gastroenteritis (salmonellosis) in humans [10]. NTS has more than 2,610 serovars, their host specificity and the ability to cause diseases varies among serovars [26]. Symptoms of salmonellosis include diarrhea, fever, stomach ache, vomiting, nausea, muscular or articular pain and loss of appetite [32], however, infection occasionally results in life-threatening systemic involvement especially to the patients with compromised immunity.

The pathogenicity of NTS is based on the expression of virulence factors which facilitate its mechanism and neutralize the host's defense through adhesion, invasion, internalization, intracellular proliferation,

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fimbrial expression, systemic infection, toxin production and survival processes [9; 26]. Multiple virulence genes are present on various locations of NTS genome including *Salmonella* pathogenicity islands (SPIs) and mobile genetic elements such as prophages and plasmids which are often disseminated among the bacterial population. These elements are conserved among *Salmonella* species while some of them could be serovar specific [15].

The genetic relatedness identification among NTS serovars recovered from different food and environmental samples is important to determine the cross contamination and the source of contamination. This is of great importance leading to the implementation of control measures. DNA-based fingerprinting techniques including enterobacterial repetitive intergenic consensus (ERIC) and repetitive extragenic palindromic-PCR method that utilizes BOX-A1R primers (BOX-PCR) are important tools for genetic discrimination of *Salmonella* isolates. The methods are rapid, highly reliable and sensitive in discriminating between closely related strains. In recent years, the ERIC-PCR fingerprinting method has been used to confirm epidemiological relationships between various isolates, and this method has shown high discriminatory power [12, 18].

In Malaysia, several studies reported the presence of NTS in salad vegetables sold in retail markets. Since salad vegetables are consumed raw, there is increasing risk of acquiring salmonellosis among consumers. Thus, the present study aimed to determine the virulence of 58 NTS isolates and their genetic relatedness.

Materials and Methods

Non-typhoidal *Salmonella* isolates

Fifty-eight NTS isolates from salad vegetables and environmental samples from previous study [14] were used. They were isolated according to the International Standard protocol ISO 6579:2002(E) [17]. The NTS strains were isolated from farms (*ulam raja*, n = 2; irrigation water, n = 5; soil, n = 1), wet markets (*ulam raja*, n = 7; *selom*, n = 13; *pegaga*, n = 13; *timun*, n = 2; *kacang botor*, n = 2; *bayam brazil*, n = 7) and supermarkets (*ulam raja*, n = 3; *selom*, n = 1; *pegaga*, n = 2). The isolates are belonged to diverse serovars including *S*. weltevreden (12), *S*. Albany (6), *S*. Hvittingfoss (5), *S*. Aberdeen (4), *S*. Poona (3) *S.* Corvallis (3), *S*. Augustenborg (2), *S*. Newport (2), *S*. Saintpaul (2), *S*. Rissen (2), *S*. Stanley (2), *S*. Lexington (2), *S*. Hindmarsh (1), *S*. Virginia (1), *S*. Bareilly (1), *S*. Virchow (1), *S*. Kentucky (1), *S*. Rubislaw (1), *S*. Muenchen (1), *S*. Agona (1), *S*. Heidelberg (1), *S*. Mountpleasant (1), *S*. Braenderup (1), Subsp. Iiib Ser 47; C; Z35 (1) and Subsp Ii Ser47:enx,z15:1,6 (1)).

DNA extraction

DNA of NTS isolates was extracted by the boiled-cell method [30]. The culture of *Salmonella* was enriched in Tryptic Soy Broth (Oxoid, CM0129, UK) by incubation at 37˚C for 18 to 24 h. Then, one ml of the enriched broth was centrifuged at 10 000 xg for 5 min to pellet the cells. The supernatant was discarded and the pellet was resuspended in 500 µl of Tris-EDTA buffer. The suspension was then boiled at 100℃ for 10 min in digital dry bath heater (MaXtableTM H10, DAIHAN, Korea) and further cooled at -20℃ for 10 min before centrifugation at 10 000 *xg* for 10 min. The supernatant containing genomic DNA of *Salmonella* was transferred to a new microcentrifuge tube and kept at -20℃ until used for subsequent PCR analysis.

Detection of virulence genes

All NTS isolates were examined by multiplex PCR for the presence of 15 genes known to be involved in *Salmonella* virulence [28]. Targeted genes and their primer sequences used are summarized in Table 1. Three sets of reaction were used to amplify the genes (set 1 amplified *spvB*, *spiA*, *pagC*, and *msgA*; set 2 amplified *invA*, *sipB*, *prgH*, *spaN*, *orgA*, and *tolC*; set 3 amplified *sitC*, *lpfC*, *sifA*, *sopB*, and *pefA*). Each set of multiplexes used the same cycling conditions and reaction mixtures, only the primers differed among the three reactions.

The PCR reaction mixture of 25 µl was prepared by the addition of 2.5 µl template DNA, 1X PCR buffer, 6 mM MgCl2, 0.03 unit of Taq DNA polymerase, 0.2 mM dNTP and 0.2 µM forward and reverse primers. The PCR reactions were performed in a thermal cycler (Veriti™, Applied Biosystems™, USA) consisted of a denaturation step for 5 min at 95˚C, followed by 30 cycles of 30 s at 94˚C, 30 s at 66.5˚C, and 2 mins at 72˚C, with a final cycle of 10 mins at 72˚C, followed by a hold at 4˚C. PCR products obtained were then subjected to horizontal gel electrophoresis in 1.5% agarose with comparison to the 100 bp DNA marker and viewed under luminescent image analyzer (LAS-4000, FUJIFILM, Japan). *Salmonella* Typhimurium ATCC 14028 was used as positive control.

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Table 1. Targeted virulence genes and primers used for amplification

No.	Gene	Primer sequence (5' → 3')[a]	Size (bp)
1	*spvB*	F: CTATCAGCCCCGCACGGAGAGCAGTTTTTA R: GGAGGAGGCGGTGGCGGTGGCATCATA	717
2	*spiA*	F: CCAGGGGTCGTTAGTGTATTGCGTGAGATG R: CGCGTAACAAAGAACCCGTAGTGATGGATT	550
3	*pagC*	F: CGCCTTTTCCGTGGGGTATGC R: GAAGCCGTTTATTTTTGTAGAGGAGATGTT	454
4	*msgA*	F: GCCAGGCGCACGCGAAATCATCC R: GCGACCAGCCACATATCAGCCTCTTCAAAC	189
5	*invA*	F: CTGGCGGTGGGTTTTGTTGTCTTCTCTATT R: AGTTTCTCCCCCTCTTCATGCGTTACCC	1070
6	*sipB*	F: GGACGCCGCCCGGGAAAAACTCTC R: ACACTCCCGTCGCCGCCTTCACAA	875
7	*prgH*	F: GCCCGAGCAGCCTGAGAAGTTAGAAA R: TGAAATGAGCGCCCCTTGAGCCAGTC	756
8	*spaN*	F: AAAAGCCGTGGAATCCGTTAGTGAAGT R: CAGCGCTGGGGATTACCGTTTTG	504
9	*orgA*	F: TTTTTGGCAATGCATCAGGGAACA R: GGCGAAAGCGGGGACGGTATT	255
10	*tolC*	F: TACCCAGGCGCAAAAAGAGGCTATC R: CCGCGTTATCCAGGTTGTTGC	161
11	*sitC*	F: CAGTATATGCTCAACGCGATGTGGGTCTCC R: CGGGGCGAAAATAAAGGCTGTGATGAAC	768
12	*lpfC*	F: GCCCCGCCTGAAGCCTGTGTTGC R: AGGTCGCCGCTGTTTGAGGTTGGATA	641
13	*sifA*	F: TTTGCCGAACGCGCCCCCACACG R: GTTGCCTTTTCTTGCGCTTTCCACCCATCT	449
14	*sopB*	F: CGGACCGGCCAGCAACAAAACAAGAAGAAG R: TAGTGATGCCCGTTATGCGTGAGTGTATT	220
15	*pefA*	F: GCGCCGCTCAGCCGAACCAG R: GCAGCAGAAGCCCAGGAAACAGTG	157

[a]F = forward; R = reverse

Genetic relatedness analysis using ERIC- and BOX-PCR

A total of 33 isolates belonging to *S*. Weltevreden, *S*. Albany, *S*. Hvittingfoss, *S*. Aberdeen, *S*. Poona and *S*. Corvallis serovars which consist of at least three isolates for each serovar were subjected to genetic diversity test by using ERIC-PCR and BOX-PCR methods. The condition for this method was in accordance with Bilung *et al*. [6] with modifications on the reagent concentration and reaction condition. In ERIC-PCR, the primer pairs used were ERIC 1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC 2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′). To prepare 25 µl of PCR mixture, 1.0 µM of ERIC 1R primer, 1.0 µM of ERIC 2 primer, 2.0 µl of DNA template, 1X PCR buffer, 0.2 mM dNTP, 2.5 mM MgCl2, and 2.0 unit of Taq DNA polymerase were mixed together. In BOX-PCR, the primer used was BOX A1R (5'-CTACGGCAAGGCGACGCTGACG-3'). To prepare 25 µl of PCR mixture, 0.4 µM of BOX A1R primer, 2.0 µl of DNA template, 1X PCR buffer, 0.2 mM dNTP, 2.0 mM MgCl2 and 2.0 unit of Taq DNA polymerase were mixed together. The amplification reactions for ERIC and BOX-PCR were carried out in a thermal cycler (Veriti™, Applied Biosystems™, USA) according to the condition presented in Table 2 and Table 3, respectively. The PCR products from ERIC- and BOX-PCR were separated in 1% agarose gel with 1 kb DNA ladder and viewed under a luminescent image analyzer (LAS-4000, FUJIFILM, Japan). The banding patterns generated were analyzed using PyElph 1.4 software program. The dendrograms were constructed using an unweighted pair group method with arithmetic mean (UPGMA).

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Table 2. PCR conditions for ERIC-PCR

PCR steps	Temperature (˚C)	Duration	Cycle
Initial denaturation	95	5 min	-
Denaturation	95	30 sec	35
Annealing	47	30 sec	
Extension	52	1 min	
Final extension	72	8 min	-

Table 3. PCR conditions for BOX-PCR

PCR steps	Temperature (˚C)	Duration	Cycle
Initial denaturation	95	5 min	-
Denaturation	95	1 min	35
Annealing	40	45 sec	
Extension	72	2 min	
Final extension	72	10 min	-

Discriminatory Index (*D*)

The discriminatory power (*D*) of ERIC- and BOX-PCR typing methods were calculated based on Simpson's Index of Diversity using the formula described by Hunter and Gaston [16]:

$$D = 1 - \left[\frac{1}{N(N-1)} \right] \sum_{j=1}^{S} nj(nj-1)$$

"*N*" denotes the total number of strains in the sample population, "*s*" denotes the total number of types described, "*nj*" denotes the number of strains belonging to the jth type. The higher the discriminatory index, the greater the effectiveness of a particular fingerprinting method to discriminate different strains, a value of 1 is highly discriminatory, and a value of 0 is not discriminatory.

Results and Discussion

Detection of virulence genes in NTS isolates

Overall distribution of virulence genes in NTS isolates and their presence according to different serovars are presented in Table 4 and Table 5, respectively. Results indicate the presence of six to 13 types of virulence genes in each of NTS isolates with 53 (91.38%) isolates carried more than 11 genes. All isolates were found to carry *msgA*, *tolC*, *sitC* and *sopB*, while none of them carried *spvB* and *pefA* genes. Results also discovered high frequency of *spvB* (93.10%), *spiA* (94.83%), *invA* (96.55%), *sipB* (98.28%), *prgH* (94.83%), *spaN* (96.55%) and *orgA* (98.28%) genes among the isolates. All 12 *S*. Weltevreden isolates (100%) carried eight genes (*spiA*, *pagC*, *msgA*, *sipB*, *orgA*, *tolC*, *sitC* and *sopB*), 11 isolates (91.67%) carried *invA*, *prgH* and *spaN*, while only one isolate also carried *lpfC* and *sifA* genes. All *S*. Albany (six isolates) carried 12 genes and only one isolate carried additional *sifA* gene. All five isolates of *S*. Hvittingfoss carried 13 types of genes.

An important trait that *Salmonella* possesses to invade, survive and multiply in a host cell to manifest the pathogenic process of salmonellosis are mainly ruled by the presence of genetic determinants for virulence. These virulence determinants have been extensively studied in *Salmonella* [5]. Based on virulence profiles in this present study, all isolates harboured at least six virulence genes with 53 (91.38%) isolates harboured more than 11 genes, thus highlighting the significant pathogenic potential of the strains. The detected genes include *pagC*, *invA*, *sipB*, *prgH*, *spaN*, *orgA*, *sifA*, *sopB*, *spiA*, *sitC* (clustered in Salmonella Pathogenicity Island), *msgA*, *lpfC* (reside in the chromosome) [2; 10] and *tolC* (located on the bacterial outer membrane) [19]. Each of these genes play an important role by working synergistically to maintain the survival of *Salmonella* within the host cell and assist the microorganism to express its virulence for successful *Salmonella* infection.

The *msgA*, *tolC*, *sitC* and *sopB* genes were identified in all 58 isolates indicating that these virulence genes are widespread in *Salmonella* isolated from salad vegetables. These results agree with previous

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studies reporting high prevalence of these genes in *Salmonella* [4; 5; 15; 26; 27; 29; 31]. *tolC* and *sopB* are important in *Salmonella* virulence which responsible to the ability of invading host intestinal epithelial cells [31]. While *msgA* is related with the survival and replication within macrophage, and *sitC* encode products for iron uptake [31].

None of the NTS isolates tested were found to carry *spvB* and *pefA* genes. Several other studies also reported absence or low frequency of these genes in *Salmonella* isolates [5; 26; 31; 29]. *spvB* and *pefA* genes are known to reside in plasmids [10]. The absence of these genes in all isolates might be due to the localization of the genes, as plasmids are present in only a few *Salmonella* serotypes [33].

More than 91.67% of *S*. Weltevreden were found to carry at least 11 virulence genes indicating the possibility of these isolates to cause salmonellosis. A study by Minh *et al*. [21] also reported the presence of virulence-associated genes in *S*. Weltevreden isolates including genes for survival at systemic sites in *Salmonella enterica*, suggesting that the strains are capable of invasion in the intestine of host organisms. *S*. Weltevreden is known as a dominant serovar associated with foodborne gastroenteritis caused by fresh vegetables intake in South-East Asia and it is emerging in Europe, this could be linked with the capability of *S*. Weltevreden to survive on plant surface as the genomes contain additional carbohydrate metabolism clusters that enable this serovar to survive in alternative habitats [7].

All *S*. Albany serovars in this study were found carried at least 12 virulence genes with one isolate carried 13 genes. This finding could be of concern as these *S*. Albany have the probability to cause disease with the presence of considerable numbers of virulence traits. Furthermore, Sabri *et al*. [25] have demonstrated the pathogenesis of *S*. Albany by experimental mice which revealed the occurrence of haemorrhagic enteritis, cecitis and colonitis as soon as 24 hours after infection.

All isolates of *S*. Hvittingfoss tested in this study were found carried 13 types of virulence genes. *S*. Hvittingfoss is a less known serovar and report of human outbreaks associated with this serovar is rare. However, in 2019, a foodborne outbreak of *S*. Hvittingfoss in China was reported affecting six peoples with clinical symptoms of diarrhea, fever, nausea, abdominal pain, dizziness and headache, furthermore five of the patients presented decreased lymphocyte and increased neutrophil percentage (high neutrophil-lymphocyte ratio) that indicate severe infection [8].

Results from this study provide more insight into the virulence characteristics of NTS strains isolated from salad vegetables and allow for establishing control plan and risk management in salad vegetable processing. It is also important to further characterize the virulence mechanisms of NTS in order to have better understanding on the pathogenicity of this organism.

Genetic relatedness of NTS isolates using ERIC-PCR and BOX-PCR

All the primers used in this study generated genomic fingerprinting patterns for all the isolates examined as depicted in Figures 1 and 2. The discriminatory indices of ERIC- and BOX-PCR at different genetic distance percentages in genotyping of 33 NTS isolates were summarized in Table 6.

The electrophoretic profile of DNA fragments obtained after ERIC-PCR amplification yielded 1-6 bands with size approximately 250 bp to 1,500 bp. From Table 6, it was summarized that ERIC-PCR clustered the isolates into 3, 6 and 9 clusters, with discriminatory indices of 0.629, 0.839 and 0.917, at genetic distance percentages of 70%, 80% and 90%, respectively. Major clusters were observed at a genetic distance percentage of 90% (Figure 1). The discriminatory index of 0.917 indicated that the overall similarity among the 33 isolates was only 8.3% due to their difference in serovar. Nine clusters were categorized at 90% genetic distance percentage. Each serovar was clustered into individual cluster (E1 – *S*. Hvittingfoss; E6 – *S*. Albany; E7 – *S*. Corvallis; E8 – *S*. Poona; E9 – *S*. Aberdeen) except for *S*. Weltevreden which were clustered into four different clusters (E2, E3, E4 and E5). One *S*. Hvittingfoss and one *S*. Weltevreden were found to be a single unique isolate.

BOX-PCR of 33 NTS isolates yielded different banding patterns between 4 and 6 bands ranging from 250 bp to 2,000 bp. Table 6 indicates that BOX-PCR clustered the isolates into 2, 2, and 7 clusters, with discriminatory indices 0.477, 0.515 and 0.873 at genetic distance percentages of 70%, 80% and 90%, respectively. It was noticed that major clusters were defined at a genetic distance percentage of 90% (Figure 2). The overall similarity among the 33 isolates was 12.7% since the discriminatory index for BOX-PCR was 0.873. *S*. Corvallis (B2) and *S*. Aberdeen (B3) were clustered into individual cluster. Besides, *S*. Albany and *S*. Weltevreden were clustered into two different clusters (B4, B5, B6 and B7). One *S*. Corvallis isolate was not grouped into any cluster.

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The effectiveness of ERIC- and BOX-PCR in a genetic relatedness study of *Salmonella* serovars had been evaluated by many researchers [11; 13; 24]. In this present study, a meaningful clustering for ERIC- and BOX-PCR were identified at a genetic distance percentage of 90% with discriminatory index (*D*) of 0.917 and 0.873, respectively. The *D* value of >0·9 is recommended by Hunter and Gaston [16] as desirable for good differentiation of isolates tested. It was found that ERIC-PCR provide higher discriminatory power and able to distinguish each different serovar of NTS tested. However, it was also observed that a few isolates were grouped into the same cluster for ERIC- and BOX-PCR such as *S*. Corvallis (E7 and B2) and *S*. Aberdeen (E9 and B3).

From the ERIC-PCR dendrogram, all *S*. Hvittingfoss, *S*. Albany, *S*. Corvallis, *S*. Poona and *S*. Aberdeen isolates were clustered into their specific serovars even though they were recovered from different samples collected at different sampling point. These findings indicate the effectiveness of ERIC-PCR to differentiate NTS isolates according to their specific serovars. Furthermore, these also might indicate the same sources of NTS that contaminate salad vegetables sold in the markets as the same local farmers were distributing their salad vegetables to all the nearby markets. Previously, there was also a report which demonstrated the success of ERIC-PCR in discriminating *Salmonella* strains from different serovars and sort out the strains from food and clinical samples into different clusters [23].

S. Weltevreden isolates were clustered into four different groups (E2, E3, E4 and E5) with one single type. All *S*. Weltevreden from wet market were isolated from wet market 2 except one isolate from wet market 3 (W3UR). The W3UR isolate was clustered into E2 group indicating the same source of the isolate with others. The E5 cluster comprises of *S*. Weltevreden from farm level (F5 and F1). Isolate from soil in farm 5 was categorized into the same cluster with isolates from irrigation water from farm 1, this finding reflects the similarity of NTS isolates from environment even though they were originated from different farm. In addition, the different farms are located in a short distance away from each other. Similarly, Abakpa *et al*. [1] also demonstrated high genetically related strains of *Salmonella enterica* recovered from soil and irrigation water samples from vegetable farms in Kano and Plateau states of Nigeria. ERIC-PCR is a useful DNA-based typing method for fingerprinting of NTS. Several investigators have demonstrated good discriminatory and typing capacity of ERIC-PCR, suggesting this method as a reliable typing tool for NTS isolates from foods and human [9; 22].

Dendrogram constructed from BOX-PCR at 90% similarity showed that this method was unable to distinguish *S*. Hvittingfoss and *S*. Poona serovars as they were categorized together in one cluster (B1). While others were clustered into their individual serovars with single isolate of *S*. Corvallis (W3BB). Analysis of fingerprints in the BOX-PCR dendrogram by Lozano-Villegas *et al*. [20] in their study also showed that the method was unsuccessful to differentiate all *Salmonella* strains at the serotype level.

All NTS isolates in this study could not be clustered based on their source of isolation and type of sample as they were found genetically diverse with no clear association. However, clustering of NTS isolates based on serotype might demonstrate the relationship among isolates of same serovars even though they were recovered from different source and different type of samples.

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Table 4. Virulence genes in NTS isolates

Isolate code[a]	*Salmonella* serovars	Virulence genes															Total genes (%)
		spvB	***spiA***	***pagC***	***msgA***	***invA***	***sipB***	***prgH***	***spaN***	***orgA***	***tolC***	***sitC***	***lpfC***	***sifA***	***sopB***	***pefA***	
FUR1	*S*. Augustenborg	A[b]	P[c]	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
FUR2	*S*. Augustenborg	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
FIW1	*S*. Weltevreden	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
FIW2	*S*. Weltevreden	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
FIW3	*S*. Weltevreden	A	P	P	P	A	P	A	A	P	P	P	A	A	P	A	8/15 (53.33)
FIW4	Subsp. Iiib Ser 47; C; Z35	A	A	A	P	P	P	P	P	P	P	P	A	A	P	A	9/15 (60.00)
FIW5	Subsp Ii Ser47:enx,z15:1,6	A	A	A	P	A	P	A	A	P	P	P	A	A	P	A	6/15 (40.00)
FSO1	*S*. Weltevreden	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
WUR1	*S*. Weltevreden	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
WUR2	*S*. Weltevreden	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
WUR3	*S*. Albany	A	P	P	P	P	P	P	P	P	P	P	P	A	P	A	12/15 (80.00)
WUR4	*S*. Albany	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WUR5	*S*. Albany	A	P	P	P	P	P	P	P	P	P	P	P	A	P	A	12/15 (80.00)
WUR6	*S*. Newport	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WUR7	*S*. Hindmarsh	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WSE1	*S*. Weltevreden	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
WSE2	*S*. Weltevreden	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
WSE3	*S*. Weltevreden	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
WSE4	*S*. Weltevreden	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
WSE5	*S*. Virginia	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WSE6	*S*. Aberdeen	A	A	P	P	P	P	P	P	P	P	P	P	P	P	A	12/15 (80.00)
WSE7	*S*. Aberdeen	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WSE8	*S*. Bareilly	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WSE9	*S*. Hvittingfoss	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WSE10	*S*. Hvittingfoss	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WSE11	*S*. Virchow	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WSE12	*S*. Saintpaul	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WSE13	*S*. Kentucky	A	P	P	P	P	P	P	P	P	P	P	P	A	P	A	12/15 (80.00)
WP1	*S*. Rubislaw	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
WP2	*S*. Rissen	A	P	P	P	P	A	A	P	A	P	P	P	A	P	A	9/15 (60.00)
WP3	*S*. Newport	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WP4	*S*. Saintpaul	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WP5	*S*. Albany	A	P	P	P	P	P	P	P	P	P	P	P	A	P	A	12/15 (80.00)
WP6	*S*. Albany	A	P	P	P	P	P	P	P	P	P	P	P	A	P	A	12/15 (80.00)
WP7	*S*. Poona	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
WP8	*S*. Poona	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
WP9	*S*. Poona	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)

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Isolate code[a]	*Salmonella* serovars	Virulence genes															Total genes (%)
		spvB	***spiA***	***pagC***	***msgA***	***invA***	***sipB***	***prgH***	***spaN***	***orgA***	***tolC***	***sitC***	***lpfC***	***sifA***	***sopB***	***pefA***	
WP10	*S*. Hvittingfoss	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WP11	*S*. Aberdeen	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WP12	*S*. Corvallis	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
WP13	*S*. Corvallis	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
WT1	*S*. Weltevreden	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WT2	*S*. Stanley	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WKB1	*S*. Stanley	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WKB2	*S*. Muenchen	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WBB1	*S*. Weltevreden	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
WBB2	*S*. Hvittingfoss	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WBB3	*S*. Albany	A	P	P	P	P	P	P	P	P	P	P	P	A	P	A	12/15 (80.00)
WBB4	*S*. Corvallis	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
WBB5	*S*. Aberdeen	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
WBB6	*S*. Agona	A	P	P	P	P	P	P	P	P	P	P	P	A	P	A	12/15 (80.00)
WBB7	*S*. Heidelberg	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
SUR1	*S*. Mountpleasant	A	A	A	P	P	P	P	P	P	P	P	A	A	P	A	9/15 (60.00)
SUR2	*S*. Braenderup	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
SUR3	*S*. Lexington	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
SSE1	*S*. Lexington	A	P	P	P	P	P	P	P	P	P	P	A	A	P	A	11/15 (73.33)
SP1	*S*. Hvittingfoss	A	P	P	P	P	P	P	P	P	P	P	P	P	P	A	13/15 (86.66)
SP2	*S*. Rissen	A	P	P	P	P	P	P	P	P	P	P	P	A	P	A	12/15 (80.00)

[a] Isolate code: F (farm), W (wet market), S (supermarket), UR (*ulam raja*), IW (irrigation water), SO (soil), SE (*selom*), P (*pegaga*), T (*timun*), KB (*kacang botor*), BB (*bayam brazil*).
[b] A: absent
[c] P: present

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Table 5. Distribution of virulence genes according to NTS serotypes

***Salmonella* serovars**	**No. of isolates**	**Virulence genes**														
		spvB	*spiA*	*pagC*	*msgA*	*invA*	*sipB*	*prgH*	*spaN*	*orgA*	*tolC*	*sitC*	*lpfC*	*sifA*	*sopB*	*pefA*
S. Weltevreden	12	0	12	12	12	11	12	11	11	12	12	12	1	1	12	0
S. Albany	6	0	6	6	6	6	6	6	6	6	6	6	6	1	6	0
S. Hvittingfoss	5	0	5	5	5	5	5	5	5	5	5	5	5	5	5	0
S. Aberdeen	4	0	3	4	4	4	4	4	4	4	4	4	4	4	4	0
S. Poona	3	0	3	3	3	3	3	3	3	3	3	3	0	0	3	0
S. Corvallis	3	0	3	3	3	3	3	3	3	3	3	3	0	0	3	0
S. Augustenborg	2	0	2	2	2	2	2	2	2	2	2	2	2	2	2	0
S. Newport	2	0	2	2	2	2	2	2	2	2	2	2	2	2	2	0
S. Saintpaul	2	0	2	2	2	2	2	2	2	2	2	2	2	2	2	0
S. Rissen	2	0	2	2	2	2	1	1	2	1	2	2	2	0	2	0
S. Stanley	2	0	2	2	2	2	2	2	2	2	2	2	2	2	2	0
S. Lexington	2	0	2	2	2	2	2	2	2	2	2	2	0	0	2	0
S. Hindmarsh	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0
S. Virginia	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0
S. Bareilly	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0
S. Virchow	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0
S. Kentucky	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	0
S. Rubislaw	1	0	1	1	1	1	1	1	1	1	1	1	0	0	1	0
S. Muenchen	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0
S. Agona	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	0
S. Heidelberg	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0
S. Mountpleasant	1	0	0	0	1	1	1	1	1	1	1	1	0	0	1	0
S. Braenderup	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Subsp. Iiib Ser 47; C; Z35	1	0	0	0	1	1	1	1	1	1	1	1	0	0	1	0
Subsp Ii Ser47:enx,z15:1,6 (1)	1	0	0	0	1	0	1	0	0	1	1	1	0	0	1	0
Total isolates (%)	58	0 (0)	54 (93.10)	55 (94.83)	58 (100)	56 (96.55)	57 (98.28)	55 (94.83)	56 (96.55)	57 (98.28)	58 (100)	58 (100)	35 (60.34)	26 (44.83)	58 (100)	0 (0)

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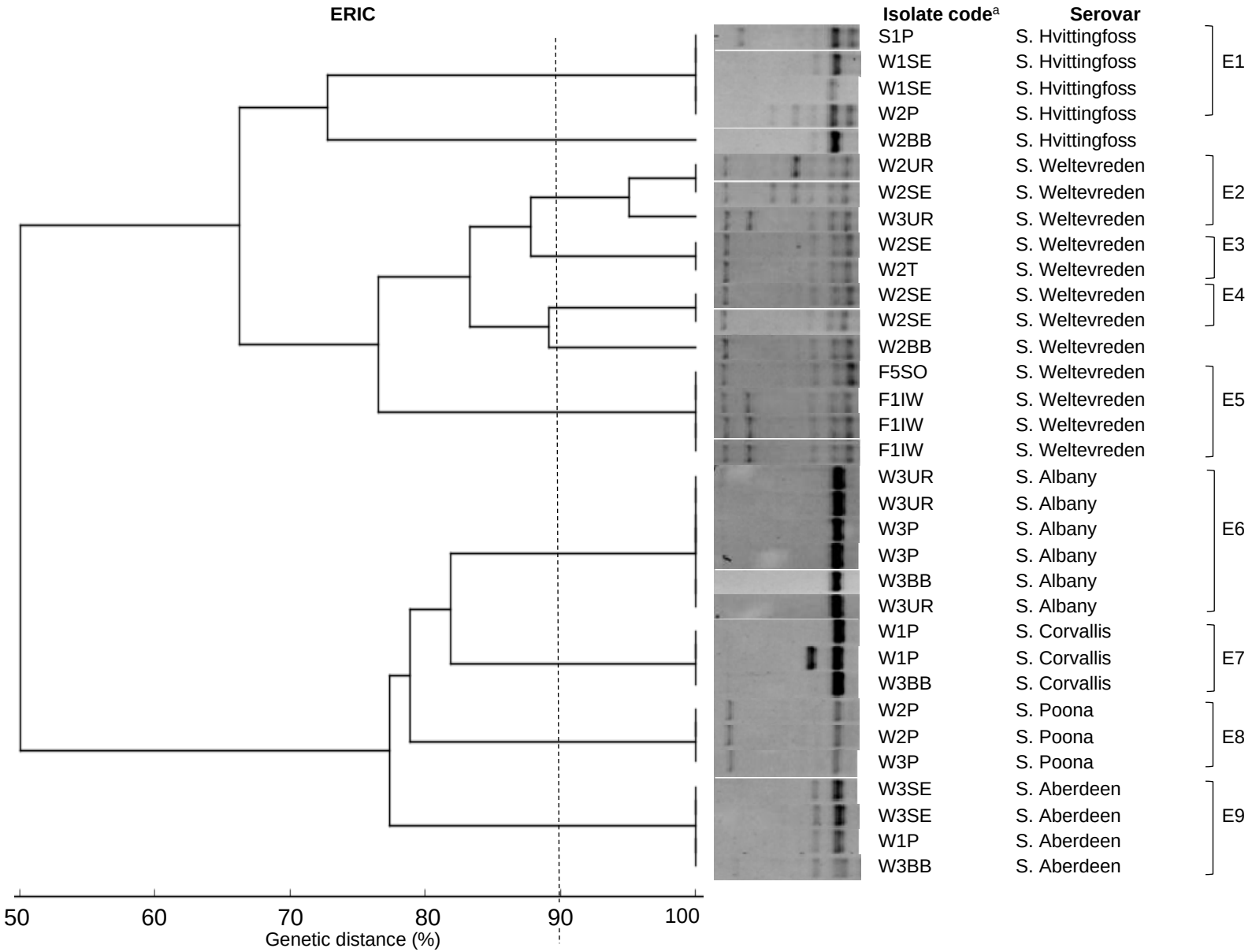

Figure 1. Dendrogram generated from ERIC-PCR fingerprinting of 33 isolates of NTS.
[a] Isolate code: F (farm), W (wet market), S (supermarket), UR (*ulam raja*), IW (irrigation water), SO (soil), SE (*selom*), P (*pegaga*), T (*timun*), KB (*kacang botor*), BB (*bayam brazil*).

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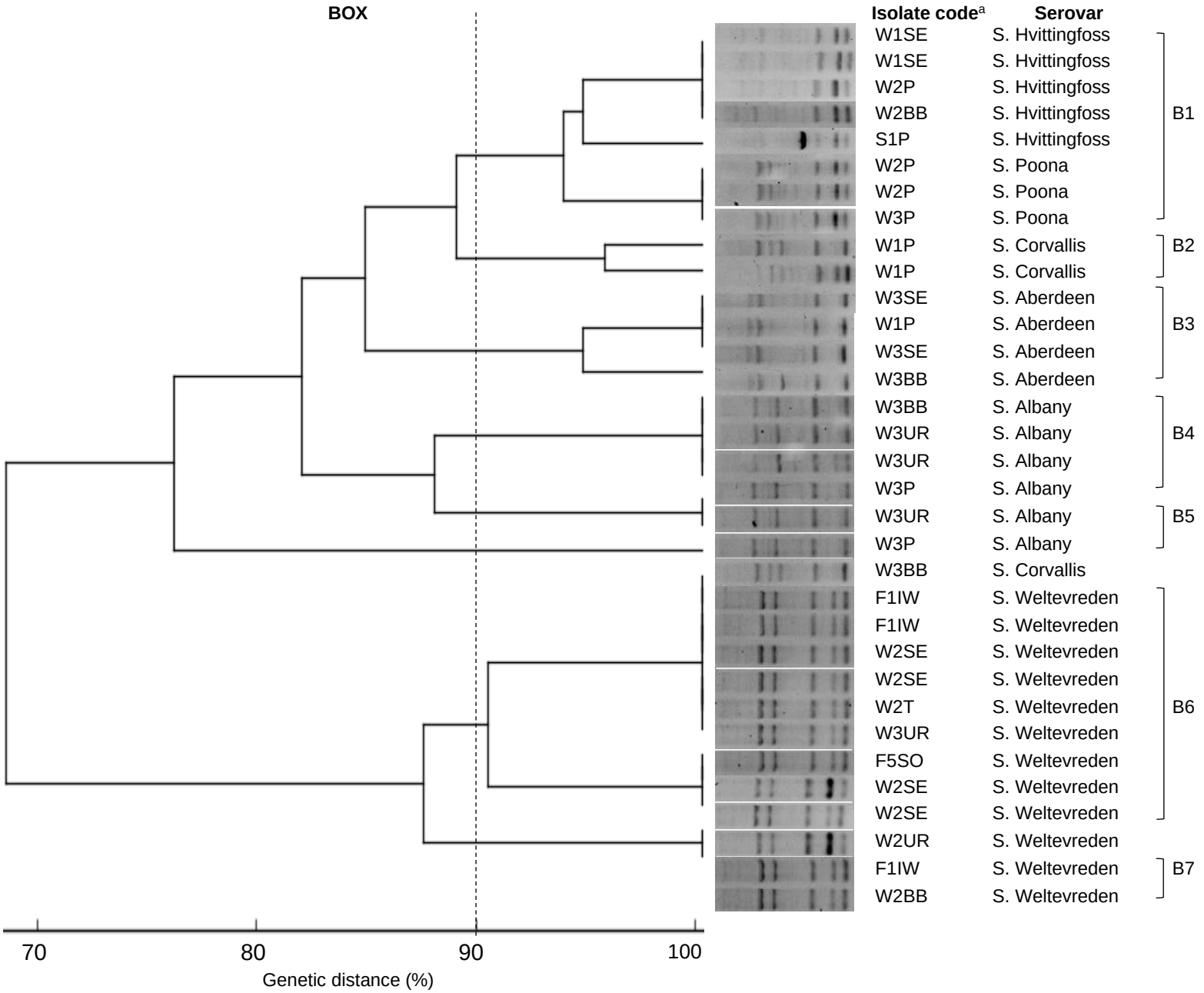

Figure 2. Dendrogram generated from BOX-PCR fingerprinting of 33 isolates of NTS. a Isolate code: F (farm), W (wet market), S (supermarket), UR (ulam raja), IW (irrigation water), SO (soil), SE (selom), P (pegaga), T (timun), KB (kacang botor), BB (bayam brazil).

Table 6. Discriminatory indices of ERIC-PCR and BOX-PCR in genotyping of NTS isolates (n = 33)

Method	Genetic distance	No. of clusters	Cluster sizes	No. of single isolate	Discriminatory index
ERIC-PCR	70%	3	5,12,16	0	0.629
	80%	6	4,8,4,9,3,4	1	0.839
	90%	9	4,3,2,2,4,6,3,3,4	2	0.917
BOX-PCR	70%	2	21,12	0	0.477
	80%	2	20,12	1	0.515
	90%	7	8,2,4,4,2,7,3,2	1	0.873

Conclusions

In this present study, we ascertained the presence of 13 types of virulence traits in NTS isolates and each isolate carried at least six traits that responsible to exhibit the pathogenicity of NTS. The ERIC-PCR typing technique had successfully differentiate all NTS isolates according to their serotype. It was also able to differentiate the NTS originated from salad vegetables and environmental sample sources. Findings from this study suggests the necessity of effective control measures to minimize the spread of NTS in order to prevent salmonellosis cases due to salad vegetables intake.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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