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High Resolution Whole Genome Multilocus Sequence Typing (wgMLST) Schemes for *Salmonella enterica* Weltevreden Epidemiologic Investigations

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Non-typhoidal *Salmonella* is one of the main pathogens causing food-borne illness in humans, with up to 20% of cases resulting from consumption of pork products. Over the gastroenteritis signs, multidrug resistant *Salmonella* has arisen. In this study, pan-susceptible phenotypic strains of *Salmonella enterica* serotype Weltevreden recovered from pig production chain in Chiang Mai, Thailand during 2012–2014 were chosen for analysis. The aim of this study was to use whole genome sequencing (WGS) data with an emphasis on antimicrobial resistance gene investigation to assess their pathogenic potential and genetic diversity determination based on whole genome Multilocus Sequence Typing (wgMLST) to expand epidemiological knowledge and to provide additional guidance for disease control. Analyis using ResFinder 3.0 for WGS database tracing found that one of pan-susceptible phenotypic strain carried five classes of resistance genes: aminoglycoside, beta-lactam, phenicol, sulfonamide, and tetracycline associated genes. Twenty four and 36 loci differences were detected by core genome Multilocus Sequence Typing (cgMLST) and pan genome Multilocus Sequence Typing (pgMLST), respectively, in two matching strains (44/13 vs A543057 and A543056 vs 204/13) initially assigned by conventional MLST and Pulsed-field Gel Electrophoresis (PFGE). One hundread percent discriminant ability can be achieved using the wgMLST technique. WGS is currently the ultimate molecular technique for various in-depth studies. As the findings stated above, a new of "gold standard typing method era" for routine works in genome study is being set.

Keywords: ***Salmonella*** **Weltevreden, pork, Thailand, pan-susceptible, whole gemone MLST**

Introduction

Non-typhoidal *Salmonella* is one of the important zoonotic pathogens that can lead to foodborne illness outbreaks worldwide [1–3]. Nearly a hundred million patients and 155,000 deaths have been reported to occurred in a single year [4, 5]. Initial symptoms of human salmonellosis are the onset of fever together with gastroenteritis signs such as vomiting, nausea, and profuse diarrhea within two days after infection [6]. Over the clinical signs, occurrence of multi-drug resistant strains is a source of serious and rising concern [7–10]. Food originating from livestock, including eggs, dairy products, and raw or under-cooked meats, are consid-

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ered as a major source of the disease [1, 3]. Pork products have been reported to be involved in 20% of cases [11]. In Chiang Mai, Thailand, there have been many studies reporting on *Salmonella* in the pig production chain over the past two decades. High prevalences of multidrug resistant *Salmonella* have been documented [12–15], proposing that *Salmonella* problems cannot be exterminated in the area.

In the studies by Tadee *et al*. [14] and Patchanee *et al*. [15], one hundred and twenty strains of *Salmonella enterica* were identified. Of those, only seven strains were included in serotype Weltevreden. Six of them were remarkably susceptible to all ten antimicrobials tested (pan-susceptible). Actually, all phenotypic appearances are the consequence from the expression of an organism's genetic code, genotype. In some condition, antimicrobial resistant genes harbored in some strains are likely unexpressed to an associated phenotype [16]. Pan-susceptible phenotype is not reflected in a low pathogenic potential for disease burden. Therefore, molecular level detection of acquired antimicrobial resistance genes is required.

Execution of *Salmonella* typing is authoritative argument for disease investigation and surveillance [17–20]. From those seven *S.* Weltevreden strains, three pulsotypes were identified by Pulsed-field gel electrophoresis (PFGE) analysis. A "farm to slaughterhouse" transmission route was proved in two closely related groups of three strains each. The remaining strain originated at the retail level was placed on a far away cluster by phylogenetic tree analysis which means that the contamination of *Salmonella* in the retail pork sample may have occurred independently. Unclean equipment, poor processing practices and storage conditions are potential contributing factors [15].

Whole genome sequencing (WGS) is a novel molecular technique which provides the opportunity to do "inside exploring" and to do "molecular typing" with clear and precise results [21]. High throughput of information on "presented" target genes can be achieved with this approach through the database scanning [22]. In addition, whole genome Multilocus Sequence Typing (wgMLST) has been recently developed as a WGS based "gene by gene" typing system [23]. Allelic variations in nucleotide sequences are defined into categorical characters from a set of many thousands of gene loci, a process which has proved very useful for strain tracking and outbreak investigation of several bacterial pathogens [24–26].

The present study used WGS data of *Salmonella* Weltevreden isolated from the pork production chain in Chiang Mai, Thailand from previous studies by Tadee *et al*. [14] and Patchanee *et al*. [15] to investigate antimicrobial resistance genes to assess the pathogenic potential jeopardizing humans. In addition, a study of the genetic diversity based on wgMLST was conducted to expand epidemiological knowledge and to provide guidance for carrying out salmonellosis control measures in the study area.

Materials and Methods

Salmonella Weltevreden strains

Samples of *Salmonella* Weltevreden circulating in the pig production chain in Chiang Mai Thailand obtained by Tadee *et al*. [14] and Patchanee *et al*. [15] studies were acquired. A total of seven strains were tested: two strains originated from pig farms, four were recovered from samples taken at pig slaughterhouses, and one was isolated from pork at a fresh food market. All strains were initially submitted to antimicrobial susceptibility testing using agar disk diffusion with a panel of ten antimicrobial agents: ampicillin (AMP) 10 μg, amoxicillin-clavulanic acid (AUG) 30 μg, sulfamethoxazole-trimethoprim (SXT) 25 μg, ciprofloxacin (CIP) 5 μg, chloramphenicol (C) 30 μg, streptomycin (S) 30 μg, nalidixic acid (NA) 30 μg, norfloxacin (NOR) 10 μg, cefotaxime (CTX) 30 μg, and tetracycline (TE) 30 μg. All underwent DNA-fingerprinting for genotype in pulsotype identification using PFGE were performed by the WHO National *Salmonella* and *Shigella* Center Laboratory (NSSC), Nonthaburi, Thailand. Characteristics of strains tested are shown in Table 1.

Whole genome sequencing

WGS data was performed at Shepperd Laboratory, Swansea University, United Kingdom. QIAamp DNA mini kits (Qiagen, Crawley, UK) were used for DNA extraction from all seven Weltevreden strains. Paired-end short read sequences of 300 bp were generated using an Illumina MiSeq genome sequencer (Illumina, Cambridge, UK). Then the multiple DNA reads in high

Table 1. Origin and characteristics of *S.* Weltevreden strains tested.

Strain ID	Where obtained[a]	Type of sample	Sampling date	Resistance pattern[b]	PFGE type
A543056	CN-farm	nipple drinker	12-Jun-12	Pan-susceptible	E1
A543057	CN-farm	nipple drinker	12-Jun-12	AMP/C/S	E2
30/13	SP-slaughterhouse	feces	26-May-13	Pan-susceptible	E2
122/13	LP-slaughterhouse	feces	9-Jun-13	Pan-susceptible	E2
44/13	SP-slaughterhouse	carcass	23-Jul-13	Pan-susceptible	E1
204/13	BET-slaughterhouse	feces	15-Sep-13	Pan-susceptible	E1
Sal9	MM-market	meat	6-Jul-14	Pan-susceptible	A4

[a]Abbreviation for the site where the sample was obtained.
[b]Antimicrobial resistance of each strain: AMP (ampicillin); C (choramphenicol); S (streptomicin).

coverage were filtered, trimmed, and assembled *de novo* using SPAdes software following the methods described by Bankevich *et al*. [20] using the reference strain of *Salmonella* Typhimurium LT2 [complete genome accession number: NC_003197]. Finally, assembled genome DNA plotter diagrams of the seven Weltevreden strains were generated using BioNumerics version 7.5.

In silico identification of putative antimicrobials resistance genes

Whole genome sequencing data of all Weltevreden strains were taken into the databases "ResFinder 3.0" at https://cge.cbs.dtu.dk/services/ResFinder/ [27]. Genes associated with the expression of aminoglycoside, beta lactam, colistin, fluoroquinolone, fosfomycin, fusidic acid, glycopeptide, macrolide, nitroimidazole, oxazolidinone, phenicols, rifampicin, sulfonamide, tetracycline, and trimethoprim resistance were explored using the default parameters (90% identification threshold with 60% minimum lenght) [22].

Multilocus Sequence Typing (MLST)

Whole genome sequencing data of the seven Weltevreden strains were characterized for allelic number determination of seven housekeeping gene loci by the Center for Genome Epidemiology (https://cge.cbs.dtu.dk/services/MLST/) [28]. The sequence type (ST) number of each Weltevreden strain with the typing results of the genes, including *aroC* (chorismate synthase), *dnaN* (DNA polymerase III subunit beta), *hemD* (uroporphyrinogenIII cosynthase), *hisD* (histidinol dehydrogenase), *purE* (phosphoribosy laminomidazole), *sucA* (alpha-ketoglutarate dehydrogenase), and *thrA* (aspartokinase I/homoserine dehydrogenase) were displayed [29].

Whole genome Multilocus Sequence Typing (wgMLST)

Allele calls with the genome assembly were determined for wgMLST analysis. BioNumerics version 7.5 (Applied Maths, Belgium) was used to carry out the core genome Multilocus Sequence Typing (cgMLST) and the pan genome Multilocus Sequence Typing (pgMLST) analysis. A set of approximately 3,000–4,000 genes located on the entire genome of each Weltevreden strain were processed. Cluster analysis of categorical values of allelic numbers for cgMLST and wgMLST were used to construct phylogenetic networks using the unweighted pair group method with arithmetic mean (UPGMA) algorithms.

Discriminatory power and concordance between genotyping methods

Simpson's diversity index represents the discriminant ability of typing techniques. The index estimates the possibility that any two individual randomly selected strains from a population will be placed into a different category. Definite uniformity and infinite diversity are ranged from values of 0 to 1, respectively [30].

The concordances among the three typing techniques were determined using the Adjusted Rand and and the Wallace coefficients, with the range from 0 to 1. High values of the Adjusted Rand coefficient points toward great a concordance between two typing techniques. An index score of 0 indicates that the two techniques do not reach agreement on any pair of elements, while a score of 1 indicates and the two partitions are exactly matching [31]. The Wallace coefficient predicts the level of con-

cordance between the results of two different techniques. A Wallace coefficient of 0 and 1 indicates that any two strains which are found to be of a similar type with one technique will have no chance and 100% chance of being grouped in a similar type when assessed by the another technique, respectively [32]. All of these were calculated using the online tool "Diversity and partition congruence coefficients calculation" at http://www.comparingpartitions.info/?link=Tool [33].

Results

Antimicrobials resistance genes profiles of *S.* Weltevreden strains

WGS data of each strain was provided to the ResFinder 3.0 database to explore for antimicrobial resistant genes (Fig. 1). Only one of the Weltevreden strains expressed a resistant phenotype (AMP/C/S). That strain, A543057, which originated from pig farm, harbored resistance genes including aminoglycoside resistance associated genes (*Add*A1, *Add*A2), a beta-lactam resistance associated gene (*bla*TEM-1B), a phenicol resistance associated gene (*cml*A1), and a sulfonamide resistance associated gene (*sul*3). The other five strains obtained at the farm and slaughterhouse which expressed pan-susceptible phenotype did not carry any resistance genes. Surprisingly, the final sample, a pan-suscepbible strain, Sal9, obtained from a pork sample at market level possessed a number of resistance genes including aminoglycoside resistance associated genes (*str*A, *Add*A1, *Add*A2), a beta-lactam resistance associated gene (*bla*TEM-1B), a phenicol resistance associated gene (*cml*A1), a sulfonamide resistance associated gene (*sul*3), and a tetracycline resistance associated gene (*tet*A).

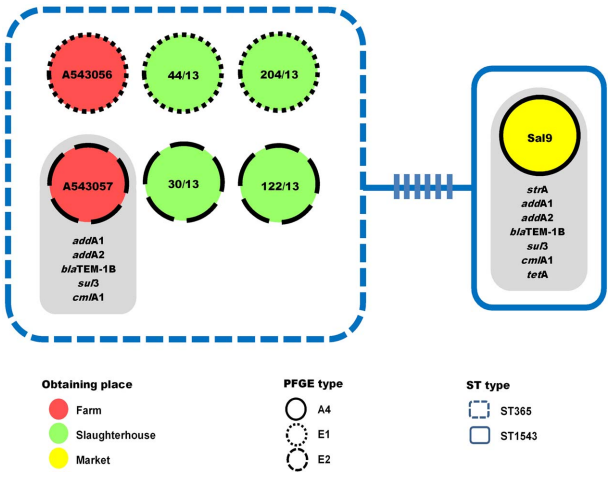

Fig. 1. Characteristics diagram of *S.* Weltevreden strains tested. Circles represent the *S.* Weltevreden strain ID. Circle color indicates the place from which the sample was taken. Circle outline style indicates the PFGE type. Square outline style indicates the ST type. Cross-hatches indicate the number of loci differences. Grey shaded areas denote the antimicrobial resistance genes harboured by each Weltevreden.

Sequence type of *S.* Weltevreden strains

Seven allelic numbers for each strain belonging a conventional MLST scheme were acquired using the WGS data approach (Fig. 1). Two sequence types of Weltevreden were identified in this study. Typing results of the six strains obtained at the farm and slaughterhouse levels were "*aroC*_130 / *dnaN*_97 / *hemD*_25 / *hisD*_125 / *purE*_84 / *sucA*_9 / *thrA*_101", assigned to ST365. The one remaining strain (Sal9) was arranged in "*aroC*_417 / *dnaN*_4 / *hemD*_15 / *hisD*_262 / *purE*_95 / *sucA*_9 / *thrA*_141", allocated to ST1543. Combining these results with the PFGE typing, two groups of 3 strains each, defined as E1 and E2 pulsotypes, were identified as ST365. Nonetheless, the remaining one belonged to the A4 pulsotype, was specified as ST1543.

WgMLST analyses of *S.* Weltevreden strains

A total of 2,491 and 4,807 genes were evaluated for core genome Multilocus Sequence Typing (cgMLST) and pan genome Multilocus Sequence Typing (pgMLST), respectively (Fig. 2). In general, in the two dendrograms the lining positions for the strains in both trees were quite analogous. The Sal9 was positioned far away from the others in both phylogenetic dendrograms with greater than 200 loci variances. In core genome analysis, only the mutual gene loci appearing in all seven Weltevreden strains were determined. The 44/13 and A543057 derived from the same ST type on diverse pulsotypes from different origin levels were found to have 24 loci differences. In the pan genome analysis, however, all gene loci were included in the examination. Two strains, A543056 and 204/13, which were obtained from differing production levels (farming vs slaughtering levels), were the most closely related strains with 36 loci differences.

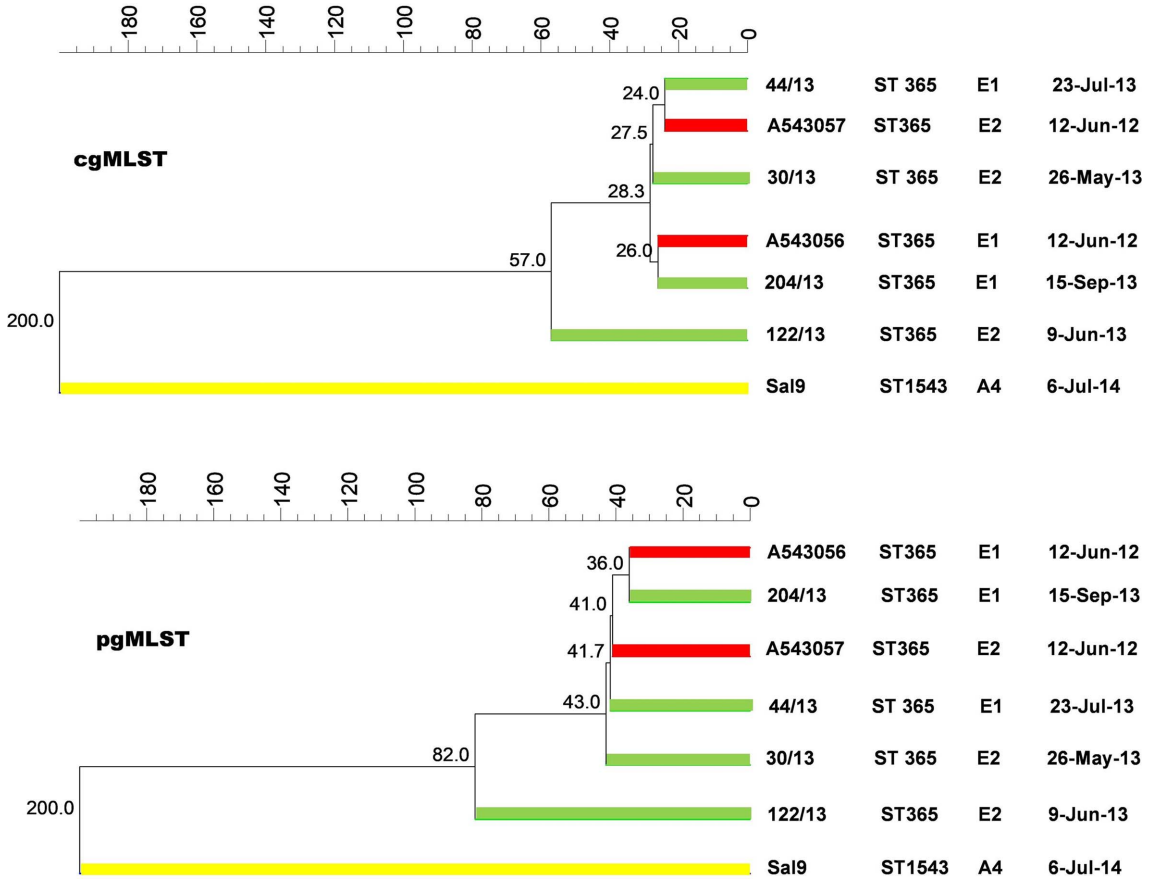

Fig. 2. Dendrogram of the core genome MLST (cgMLST) and the pan genome MLST (pgMLST) of *S.* Weltevreden strains tested. Numbers at the nodes are the numbers of loci differences in the branches, e.g., 200 indicates more than 200 locus differences. Branch color denotes the level at which the sample was obtained: red = farm; green = slaughterhouse; yellow = market). ST type, pulsotype, and sampling date are shown following each strain ID. For cgMLST and pgMLST, 2491 and 4,807 genes wer analyzed, respectively.

Discriminatory power of and concordance among three molecular typing techniques

The Simpson's diversity index, the Adjusted Rand coefficient, and the Wallace coefficient of the three molecular techniques, PFGE, MLST and wgMLST, are displayed in Table 2. The Simpson's diversity index represents power of differentiation between related strains and non-related strains with each technique. The highest chance of distinguishing all Weltevreden strains tested was found with wgMLST (1.00), followed by

Table 2. Simpson's diversity index, Adjusted Rand and Wallace coefficients, and the 95% confidence intervals of three molecular techniques (PFGE, MLST and wgMLST) in typing *S.* Weltevreden strains.

Typing technique	Simpson's index (95% CI)	Adjusted Rand coefficient (95% CI)			Wallace coefficient (95% CI)		
		PFGE	MLST	wgMLST	PFGE	MLST	wgMLST
PFGE	0.71		0.28	0.00		1.00	0.00
	(0.57-0.85)		(0.00-0.88)	(0.00-0.00)		(1.00-1.00)	(0.00-0.00)
MLST	0.28	0.28		0.00	0.40		0.00
	(0.15-0.43)	(0.00-0.88)		(0.00-0.00)	(0.15-0.65)		(0.00-0.00)
wgMLST	1.00	0.00	0.00		1.00	1.00	
	(1.00-1.00)	(0.00-0.00)	(0.00-0.00)		(1.00-1.00)	(0.64-1.00)	

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PFGE (0.71), and MLST (0.28).

Adjusted Rand and Wallace coefficients were calculated for quantitative assessment of concordances between the three molecular typing techniques. The Adjusted Rand coefficient for PFGE and MLST was 0.28, indicating a low congruence between those two techniques. There was no concordance either between PFGE and wgMLST or between MLST and wgMLST typing results. The Wallace coefficient of PFGE to MLST was 1.00, indicating the 100% probability of any two strains assigned to a certain PFGE type were assigned to be the same ST. The results were similar for the pairwise tests between wgMLST to PFGE as well as for wgMLST to MLST. In contrast, the Wallace coefficient of MLST and PFGE was 0.40. That is, the strains belonging from an identical ST had only a 40% chance of being identified as the same PFGE type. Since the wgMLST technique can distinguish all Weltevreden strains, therefore the Wallace index of PFGE to wgMLST, and MLST to wgMLST were represented in the zero value.

Discussion

In this study, various aspects of seven *Salmonella* Weltevreden strains were described. This constitutes an uncommon serotype recently found in the pig production chain in northern Thailand [13–15] and the studies attributed elsewhere [3, 34–36]. Contrast to study of Padungtod and Kaneene (2006), strains recovered from the pig production chain in the same area during 15 years ago, Weltevreden was detected as the main serotype [12]. Temporal change could have played a role in this serodistribution shift [19].

The occurrence of antimicrobial resistant bacteria is becoming the greatest concern of both of veterinary medicine and public health globally [10]. Limited treatment choices and reduction of first line empirical cures are the consequence [7–9]. In this study, five classes of antimicrobial resistance genes are found from ResFinder, including aminoglycoside, beta-lactam, phenicol, sulfonamide, and tetracycline resistant associated genes. Thus, streptomycin, ampicillin, sulfonamide, tetracycline, and antimicrobials grouped in the same class are not recommended for *Salmonella* treatment [5, 8, 10]. The wide use of sub-therapeutic dosage and extra-label usage could be a factor in the high rates of resistance [2]. As chloramphenicol has been banned from use in animal production in Thailand for decades, it may seem to be an inappropriate target to focus on. Nevertheless, "pig to human" disease transmissions are usually to be noticed. However, phenicol resistant *Salmonella* may be a problem in some patients as that drug has been widely used in human medicine [36].

The gene profiles of each strain show that two out of the seven Weltevreden strains harbored different classes of resistant genes: A543057 and Sal9 harbored four and five, respectively. The sulfonamide associated gene was carried by A543057 which was identified in the sulfonamide susceptible phenotype. In addition, five classes of resistant genes were detected in the pan-susceptible strain, Sal9. Lack of any habitation-related selective pressure may explain the circumstances which resulted in our findings [16]. However, over an extended period, the level of gene expression may increase even when occurring of any selective pressure through environmental conditions change. In practice, phenotypic resistance test results are usually only medically applied. This appears to be one reason that antimicrobial recommendations based on laboratory experiments are not effective against some bacterial strains [38]. This suggests that phenotypic and genotypic antimicrobial susceptibility tests should be conducted in parallel.

Molecular typing techniques have been developed which are essential for organism tracing in disease surveillance and outbreak investigation [18, 19]. The fingerprint-based method, Pulsed-field Gel Electrophoresis (PFGE), has emerged as the "gold standard" for bacterial typing. That technique generates DNA alignment of the whole bacterial genome digesting by restriction endonuclease of rared specific sites [17]. It has sufficient power to determine the organism attribution and to discriminate among outbreak strains. However, the process is time-consuming and labor-intensive [39]. Conventional Multilocus Sequence Typing (MLST) is implemented to nomenclature for bacterial clones using the seven targeted sequence-conserving housekeeping genes. Expanding the global relatedness of epidemiological studies can be accomplished relatively rapidly and with high reproducibility [18, 19]. Unfortunately, the low discriminatory power of MLST makes that technique less useful in the analysis of some bacterial strains [17]. To overcome those limitations, wgMLST has been suggested as a way

to extend the conventional MLST concept to the entire genome [39]. All housekeeping genes as well as genes encoding for specific functions such as virulence and resistance are included in the process. In cgMLST, all mutual gene loci which appear in at least 95% of all strains are selected for inclusion in the analytical process. Gene loci which appear in at least 5% of all samples are targeted for pgMLST study [26, 29]. Accordingly, knowledge of the entire genome sequence has been adding to international surveillance with the added strength of greater discriminant ability [23].

Quantitative assessment of the level of correspondence among the three molecular techniques in examining the same strains has been conducted. WgMLST (both cgMLST and pgMLST) was judged to be the most useful technique. One hundred percent discriminant ability can be achieved with any strain using wgMLST as measured by Simpson's index, while the Simpson's index value for PFGE and MLST were 0.71 and 0.28, respectively. Two groups with three strains each and a group of five strains could not be distinguished using the PFGE and MLST, respectively. There was no equivalence between wgMLST results and the two conventional typing techniques, i.e., there was a zero Adjusted Rand coefficient indicating no concordance between those pairwise comparisons [31]. The Wallace coefficient indicates the probability that strains assigned to the identical type using one technique will also be categorized in the same type using another technique. The Wallce coefficient for wgMLST to PFGE and for wgMLST to MLST were determined to be 1, indicating that any strains determined to belong to a similar type by wgMLST will be classified 100% of the time as the same type when evaluated using either PFGE or MLST [32].

Results of efforts to verify the "farm to slaughterhouse" transmission route based on the PFGE results of those 7 *Salmonella* Weltevreden strains tested have been inconsistent. Strains with identical PFGE patterns are assigned to different types when analyzed using the cgMLST and pgMLST methods. A common ancestor or clonal lineage from the same root with point mutations of A543056, A543057, 30/13, 44/13 and 204/13 are inferred [23]. On the other hand, in the case off interpretation of the remaining position on a far away cluster, Sal9, the results conventional typing scheme agreed with the wgMLST method. This finding suggests the *Salmonella* contamination in the pork samples from the fresh food market occurred independently and was not the result of changes which occurred at earlier levels of the production chain. Unclean equipment, improper hygienic practices, or inappropriate storage conditions are possible contributing factors [15].

In summary, WGS is an alternative molecular technique which provides in-depth scientific information. High resolution trend studies and epidemiological evidence can be delivered using that novel technique. Generating of an entire sequence is not a challenge with WGS. Extracting of necessary material matched with the study proposes is the first considered point. The findings of this study highlight that antimicrobial pan-susceptible *S.* Weltevreden can harbor various classes of resistance genes, indicating the important pathogenic potential which has resulted in failure to cure disease. Additionally, strains grouped in clonal relations using conventional typing techniques are found not to be clustered when analysis is performed using the WGS. The findings described above suggest that a new "gold standard" method for routine genome study is being established.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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