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Salmonella enterica serotype typhimurium and *S*. Stanley differ in genomic evolutionary patterns and early immune responses in human THP-1 cell line and CD14$^+$ monocytes

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ABSTRACT

Salmonella Typhimurium and *S.* Stanley are the most prevalent serogroup B serovars to infect humans in Taiwan. The aim was to determine possible factors to influence the prevalence between S. Typhimurium and S. Stanley. Genotypes were determined by pulsed field gel electrophoresis (PFGE) analysis and the intracellular survival, phagocytosis, reactive oxygen species (ROS) production of human monocyte THP-1 cell and tumor necrosis factor-α(TNF-α), interleukin-6 (IL-6), and IL-1βexpression in peripheral blood CD14$^+$ cells after infection were analyzed. 182 S. Stanley was clonal disseminated with main pulsotypes 2 from 2004 to 2007. Overall *S.* Typhimurium evolved more genotypes, while *S.* Stanley conserved in genotypes. Human blood CD14$^+$ monocytes expressed TNF-α, IL-6 and IL-1β differently among serovars and bacterial conditions (live *vs.* killed). Live *S.* Stanley and *S.* Typhimurium suppressed the TNF-α and IL-6 expression compared to killed bacteria. However, live *S.* Typhimurium stimulated more IL-1β expression than the killed bacteria, but *S.* Stanley expressed similar IL-1β levels in both conditions. Furthermore, *S.* Stanley and *S.* Typhimurium differed in intracellular survival in the THP-1 cells, an early decrease for *S.* Stanley, not for *S.* Typhimurium. Additionally, higher reactive oxygen species (ROS) production in THP-1 cells was found agsinst *S.* Stanley infection, not found in *S.* Typhimurium. However, some isolates of *S.* Stanley could recover from early loss to become more in the monocytes than *S.* Typhimurium. Difference in phagocytized number, intracellular survival, ROS production and IL-1β expression may contribute to prevalence different between two serovars.

1. Introduction

Non-typhoid *Salmonella* species are zoonotic pathogens to cause salmonellosis in humans through the consumption of contaminated foods and related products. *Salmonella* species have incorporated several chromosomal *Salmonella* pathogenicity islands (SPIs) [1,2], that include SPI-1 and SPI-2, respectively, for the invasion of epithelial cells [3,4] and intracellular survival in macrophages [5–7]. In human, *S.* Choleraesuis, *S.* Enteritidis, *S.* Typhimurium, and *S.* Typhi were more invasive to cause bacteremia, while *S.* Weltevreden, *S.* Stanley, *S.* Anatum, and *S.* Rissen were the most common serovars in stool samples from patients [8]. Of the two most prevalent serogroup B serovars in human, *S.* Typhimurium causes more extra-intestinal infections [9], while *S.* Stanley is typically less invasive [10], most commonly infects infants [11], causes diarrhea in human and can be found from food [12], including pork, chicken meat and lettuce in Tailand [13] and retail aquaculture products in Shanghai, China [14], live poultry, carcases, waste water, and soil around processing plants [15], amphibians from farm animals, urban area animals and protected area animals [16]. The prevalence of *S.* Stanley differs by sources and countries. *S.* Stanley can be disseminated by travelor [17].

S. Choleraesuis, *S.* Stanley, and *S.* Typhimurium are the most resistant serovars observed with different antigiograms in Thailand [11], with resistance such as to ampicillin, chloramphenicol, nalidixic acid,

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sulfamethoxazole-trimethoprim (SXT), and tetracycline was observed for *S.* Stanley from the animal origin [13], at least to one antimicrobial drug including sulfonamides, tetracycline, streptomycin, ampicillin and nalidixic acid for the isoaltes from aquatic products [14]. Multiple drug resistant (MDR) isolates showed resistance to nalidixic acid, streptomycin, to ampicillin, tetracycline, and SXT [15].

Entrance of *Salmonella* stimulates monocyte aggregation in Peyer's patches and mesenteric lymph nodes [18] and secreted cytokines stimulate the differentiation of monocytes into macrophages and dendritic cells [19]. Interaction of Toll-like receptors (TLRs) on macrophages with pathogen-associated molecular patterns (PAMPs) on *Salmonmella* activates the release of pro-inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) to inhibit systemic infection and promote cytokine expression. Furthermore, supernatants of Salmonella-infected nacrophage induce the interferon-γ (IFN-γ) production in human CD56$^+$ cells, natural killer (NK) cells, dependent on IL-23 and IL-1β, not IL-12 [20]. Additionally, *S.* Typhimurium can suppress intracellular SPI-2-regulated ROS production by preventing NADPH oxidase assembly at the phagosomal membrane [21].

In Taiwan, *S.* Typhimurium and *S.* Stanley were the two most prevalent serogroup B serovars to infect humans from 2004 to 2012 [22]. However, the prevalence differed more than 10% between these two. The aim of this study was to elucidate possible immune factors that influence the prevalence of *S.* Typhimurium and *S.* Stanley infection in humans.

2. Materials and methods

2.1. Bacterial isolates

In total, 182 human *S.* Stanley isolates from 2004 to 2007 were collected from 19 medical centers and district hospitals located in Taiwan and identified with antisera by the *Salmonella* Reference Laboratory of the Centers for Disease Control (CDC), Department of Health and Welfare, Taiwan, ROC. Other *Salmonella* isolates for immune analysis included *S.* Typhimurium wild-type OU5045, its derivatives OU5046, OU5048, OU5080, OU5193, human isolate 23 [23], goose isolates with different phage types [24], *S.* Enteritidis OU7130, human-specific serovar *S.* Typhi OU7049 and control strains *E. coli* strains ATCC 8739 and pir116 (Table 1).

Table 1
Characterization of bacterial strains for pathogenic study.

Species or serovars	Strains	Virulence plasmid	Note
S. Typhimurium	OU5045	+	Wild type
	OU5046	–	Virulence plasmid-less strain derived from OU5045
	OU5080	pOU115[a]	OU5046 carrying pOU115
	OU5193	pOU115[a]	OU5048 carrying pOU115
	CGC18[b]	+	Goose isolate phage type DT104
	CGC25[b]	+	Goose isolate phage type 12
	CGC28[b]	+	Goose isolate phage type U302
	235[c]	+	Human isolate phage type DT120
S. Stanley	001		Human isolate
	028		Human isolate
	036		Human isolate
	097		Human isolate
	103		Human isolate
S. Typhi	OU5049		Human isolate FR57
Escherichia coli	ATCC8739		
	pir116		

[a] Virulence plasmid from OU5045 was inserted with Tn*5*.

[b] Strains from Yu et al. [23].

[c] Strains from Chiu et al. [22].

Table 2
PCR primer sequences and PCR conditions.

Gene	Primer	Primer sequences (5'→3')	Ta (°C)	Product size (bp)
$bla_{CMY\text{-}2}$	AmpC-F	CTgACAgCCTCTTTCTCCACA	56	1005
	AmpC-R	CTACgTAgCTgCCAAATCCAC		
bla_{DHAM}	DHAM-F	AACTT TCACA ggTgT gTgCT gggT	61	405
	DHAM-R	CCgTA CgCAT ACTgg CTTTg C		
bla_{TEM}	TEM-F	gAAgA TCAgT TgggT gCACg AgT		520
	TEM-R	CAACT TTATC CgCCT CCATC CAgT		
bla_{SHV}	SHV-F	AACgg AACTg AATgA ggCgC T		141
	SHV-R	TCCAC CATCC ACTgC AgCAg CT		
$bla_{CTX\text{-}M14}$	CTX-M14-F	TACCg CAgAT AATAC gCAgg Tg		355
	CTX-M14-R	CAgCg TAggT TCAgT gCgAT CC		
$bla_{CTX\text{-}M3}$	CTX-M3-F	AATCA ACTgC CgCCA gTTCA CgCT		479
	CTX-M3-R	gAACg TTTCg TCTCC CAgCT gT		

2.2. Antimicrobial susceptibility testing and examination of bla genes

Resistance to ampicillin, ceftazidime, ceftriaxone, cefotaxime, chloramphenicol, nalidixic acid, and trimethoprim/sulfamethoxazole was determined using antimicrobial discs from Becton Dickinson and Company (Sparks, Maryland, USA) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [25] with *E. coli* ATCC 25922 as the reference. To check the genes responsible for resistance to ampicillin, ceftazidime, ceftriaxone, and cefotaxime, $bla_{CMY\text{-}2}$, bla_{DHAM}, bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M14}$, and $bla_{CTX\text{-}M3}$ genes were identified by single and multiple PCR reactions using the primers listed in Table 2.

2.3. Genotyping by pulsed-field gel electrophoresis (PFGE)

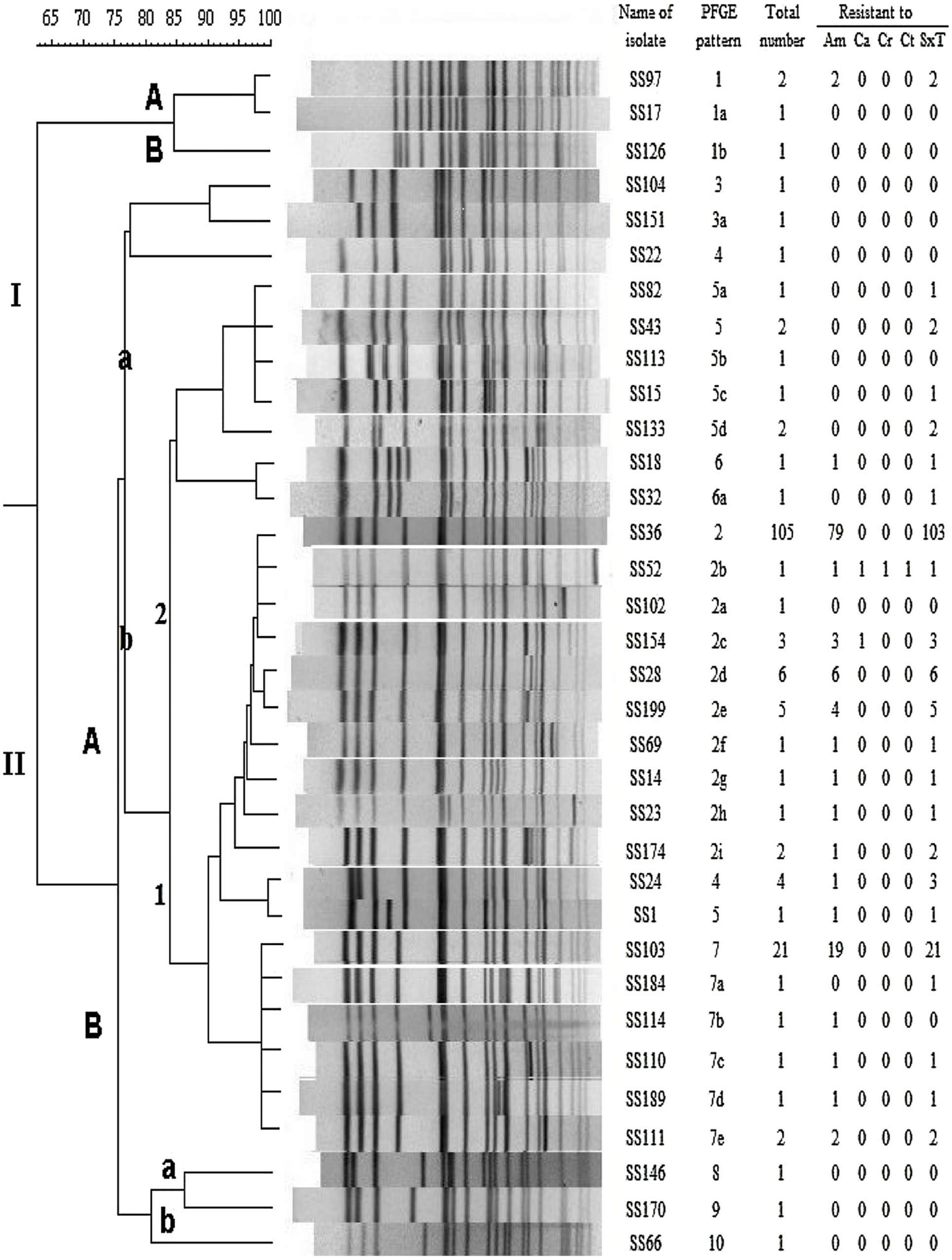
Genotypes of *S.* Stanley isolates were determined using the PulseNet standardized laboratory PFGE protocol [26]. Phylogenetic analysis was conducted by the unweighted pair group method with an arithmetic mean algorithm (UPGMA) and dice-predicted similarity value of two *Xba*I-digested PFGE patterns with 1.0% optimization and 0.7% tolerance settings. A unique PFGE pattern was defined at least one DNA band differing between the two isolates.

2.4. Cell and bacterial culture

The THP-1 cells were routinely incubated in RPMI 1640 medium (GIBCO, ThermoFisher Scientific, Taiwan) supplemented with 10% bovine serum, 10 mM HEPES buffer, 2.5 mM sodium pyruvate, 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin at 37 °C and 5% CO_2. For the phagocytosis and intracellular survival tests, 50 μM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Taiwan) was added to differentiate THP-1 cells (5×10^5 cells per well) at 37 °C for 48 h. Five *S.* Stanley isolates, six *S.* Typhimurium isolates, and *E. coli* ATCC 8739 were incubated, respectively, at 37 °C for 12 h and then centrifuged at 3000 rpm (KUBOTA 2010, Kubota, Japan) for 10 min. The pellets were washed with PBS several times and finally resuspended in PBS and $OD_{550\ nm}$ of the solutions was measured using a Bio-Rad SmartSpec™ 3000 (approximately 8.6×10^8 colony formation unit (CFU) per mL at $OD_{550\ nm} = 1$). For phagocytosis and intracellular survival, and ROS assays, three independent experiments each with at least two repeats were conducted.

2.5. Intracellular survival in THP-1 cells

Bacteria were labeled with a probe to track intracellular accumulation as described previously [27]. Briefly, 1 mL of bacterial suspension (˜1×10^8 bacteria) was stained with 20 μL of 3,3-dioctadecyloxacarbocyanine perchlorate (DioC18) at 37 °C under 5% CO_2 for 40 min. The mixture was centrifuged at $3000 \times g$ for 10 min, and the pellet was then washed with one mL PBS. This step was repeated several times



Name of isolate	PFGE pattern	Total number	Resistant to Am	Ca	Cr	Ct	SxT
SS97	1	2	2	0	0	0	2
SS17	1a	1	0	0	0	0	0
SS126	1b	1	0	0	0	0	0
SS104	3	1	0	0	0	0	0
SS151	3a	1	0	0	0	0	0
SS22	4	1	0	0	0	0	0
SS82	5a	1	0	0	0	0	1
SS43	5	2	0	0	0	0	2
SS113	5b	1	0	0	0	0	0
SS15	5c	1	0	0	0	0	1
SS133	5d	2	0	0	0	0	2
SS18	6	1	1	0	0	0	1
SS32	6a	1	0	0	0	0	1
SS36	2	105	79	0	0	0	103
SS52	2b	1	1	1	1	1	1
SS102	2a	1	0	0	0	0	0
SS154	2c	3	3	1	0	0	3
SS28	2d	6	6	0	0	0	6
SS199	2e	5	4	0	0	0	5
SS69	2f	1	1	0	0	0	1
SS14	2g	1	1	0	0	0	1
SS23	2h	1	1	0	0	0	1
SS174	2i	2	1	0	0	0	2
SS24	4	4	1	0	0	0	3
SS1	5	1	1	0	0	0	1
SS103	7	21	19	0	0	0	21
SS184	7a	1	0	0	0	0	1
SS114	7b	1	1	0	0	0	0
SS110	7c	1	1	0	0	0	1
SS189	7d	1	1	0	0	0	1
SS111	7e	2	2	0	0	0	2
SS146	8	1	0	0	0	0	0
SS170	9	1	0	0	0	0	0
SS66	10	1	0	0	0	0	0

Fig. 1. Dendrograms were constructed using pulsed field gel electrophoresis-XbaI patterns to determine the genotypes of 182 clinical *Salmonella* Stanley isolates using the unweighted pair group method with arithmetic mean (UPGMA) and dice-predicted similarity value of two patterns. The BioNumerics version 4.5 statistics program was used with 1.0% optimization and 0.7% tolerance settings.

until no unbound dye remained in the wash solution. The final supernatant was used as background check. With centrifugation at 1000 x g for 5 min after mixing 1×10^7 DioC18-coated bacteria and 1×10^6 PMA-induced THP-1 cells at multiplicity of infection (MOI) of 10, the mixture were coclutured for 3 h at 37 °C and 5% CO_2. The pellets were washed with PBS twice to remove extracellular bacteria. The THP-1 cells were resuspended in one mL PBS and the FL1 fluorescence of 1×10^4 THP-1 cells containing *Salmonella* were measured by flow cytometry (FACScan, BD) and the data was analyzed using WinMDI software.

Next, we did intracellular survival of the bacteria without labeling DioC18 [28]. The cells and bacteria were cocultured as above for 15 min. The extracellular bacteria were killed by gentamicin at final concentration of 50 μg/mL for 1 h (corresponding to the 0 h). The cells were collected at 0, 2, 4, 6, 8, 12, and 24 h and washed twice with PBS. After lysing with 0.5 mL of 1% sodium deoxycholate (Sigma-Aldrich), 100 μL aliquots of the solution were plated on Luria-Bertani (LB) plates. Viable bacteria were counted for each plate after incubation at 37 °C for 24 h.

2.6. ROS production of the THP-1 cells after infection

ROS production was measured as previous method [29]. Briefly, 2 mL of PBS containing 1×10^7 bacteria and 1×10^6 THP-1 cells (MOI = 10 and centrifugation after mixing) were incubated at 37 °C under 5% CO_2 for 30 min, and then DCFH-DA (Sigma-Aldrich) was added at a final concentration of 5 μM. The mixture was incubated for another 20 min. The unbound DCFH-DA was removed by centrifugation at 2,000 rpm for 5 min (KUBOTA 2420). The pellets were washed with two mL of PBS and then centrifuged at 2,000 rpm for 5 min. Propidium iodide (PI, Sigma) was added into the solution to reach a final concentration of 5 μg/mL. The FL1 fluorescence of the cell solution and was measured by flow cytometry and the FL1 fluorescence of 1×10^4 cells without PI staining was used as control. The data was analyzed using WinMDI software.

2.6.1. *Cytokine expression of human peripheral CD14$^+$ monocytes*

This work was approved by the ethical review board of the Tri-Service General Hospital National Defense Medical Center (no.100-05-028). Fresh human blood (500 mL) was obtained from the Taiwan Blood Services Foundation (Tainan, Taiwan R.O.C.) and peripheral CD14$^+$ monocytes were purified using human CD14$^+$ MicroBeads (Cat. 130-050-210) according to the manufacturer's instructions (Miltenyi, Biotech Inc., Auburn, CA, USA). After incubation of the CD14$^+$ monocytes in RPMI 1640 medium for 24 h, *E. coli* and various live and heat-killed *Salmonella* serovars were added, respectively, with MOI of 100 without centrifugation after mixing and incubated for 2 h. Proinflammatory cytokines TNF-α, IL-6, and IL-1β concentrations were measured in the supernatant using an ELISA kit (R&D Systems, Inc.; Minneapolis, MN, USA). Two independent experiments with three repeats were performed.

2.7. *Statistical analysis*

The differences between the serovars were analyzed using Duncan's DMRT method with SAS software. Value ($p < 0.05$) was regarded as statistically significant.

3. Results

3.1. *Antimicrobial resistance, bla genes, and PFGE genotyping of 182 S. Stanley isolates*

Among 182 isolates tested, 172 isolates were MDR and 10 isolates were sensitive to all antimicrobials tested. The resistance rate was greater than 90% for chloramphenicol and trimethoprim-sulfamethoxazole, approximately 70% for ampicillin, and less than 1% for ceftazidime, ceftriaxone, and cefotaxime. The most common antibiogrm (67.6%, 123/182) showed resistance to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole. Among the five *bla* genes examined, only the *bla*$_{\text{TEM}}$ and *bla*$_{\text{CMY-2}}$ genes were detected, and *bla*$_{\text{CMY-2}}$ was only observed in six isolates with reduced susceptibility to ceftriaxone.

PFGE analysis separated these clinical *S.* Stanley isolates into clusters I and II, each with subclusters A and B (Fig. 1). Most isolates belonged to cluster II (178 isolates) and subcluster IIA (175 isolates). Two major clones were pulsotypes 2 (131 isolates) and 7 (27 isolates) mostly in clusters IIAb1 with resistance to ampicillin and SXT and distributed evenly throughout Taiwan without change in annual prevalence. Next, we investigated the immune responses of human THP-1 cells and CD14$^+$ monocytes against infection by the two most prevalent genotypes of *S.* Stanley and the different *S.* Typhimurium phage types and genotypes.

3.2. *Intracellular survival of S. Stanley and S. Typhimurium and ROS production in the THP-1 cells*

First, we investigated the intracellular survival between two serovars in THP-1 cells. Compared to elimination of *E. coli* at 6 h, whereas all isolates of both serovars survived differently dependent on strains (Fig. 2). Using the viable bacteria at 0 h as the baseline for phagocytosis, THP-1 cells phagocytized more *S.* Stanley than *S.* Typhimurium. Further, we observed three intracellular survival patterns for *S.* Typhimurium: (1) continuously increasing intracellular numbers for wild-type OU5045, CGC25, and CGC, (2) decreasing at 2 h and increasing at 4 h for wild-type CGC18 and pSTV-less OU5046, and (3) maintaining constistent number from 0 to 4 h and increasing at 6 h for wild-type 235. On the contrary, the intracellular numbers of all *S.* Stanley isolates decreased at 2 h and then increased from 4 h onwards.

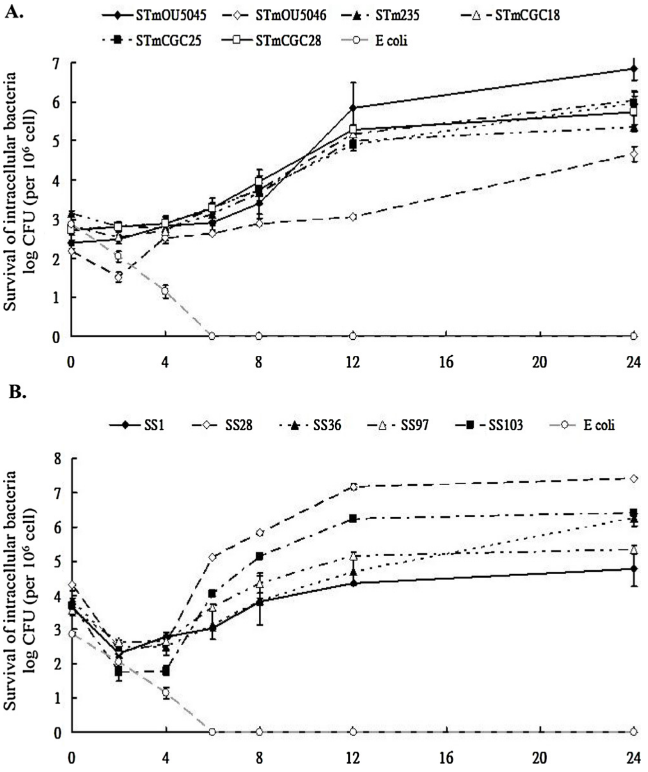


Fig. 2. Intracellular survival of different *Salmonella* Typhimurium (A) and *S.* Stanley (B) isolates in 1×10^6 THP-1 cells at different times. The x-axis indicates hours post-infection and the y-axis indicates log10 cfu from 1×10^6 THP-1 cells. *S. aureus* and *Escherichia coli* were used as controls. The value was calculated by 3 independent experiment each with two repeats.

Next, we used DioC18-labelled bactria to measure the intracellular survival between two serovars and the percentage of THP-1 cells containing intracellular bacteria by flowcytometry analysis after 3-h incubation, the turning point for *S.* Stanley to increase the intracellular bacteria. A significant difference in total fluorescence intensity was observed between two serovars, but not found among isolates in each serovar (Fig. 3A). The differences in total fluorescence intensity indicate that more THP-1 cells harbored *S.* Stanley than *S.* Typhimurium (Fig. 3B). ROS production of THP-1 cells differed between two serovars with an increase in the ROS production for *S.* Stanley and a decrease in ROS production for *S.* Typhimurium particularly the lowest decrease for strains OU5045 and OU5046 (Fig. 3C). However, ROS generation was observed not significantly among isolates within each serovar.

3.3. *The differences in cytokine expressions of human monocytes against important human serovsrs*

Cytokine synthesis/release rates of human CD14$^+$ monocytes were determined to evaluate the ability of heat-killed and alive *S.* Typhi, *S.* Enteritidis *S.* Typhimurium serovars, and *S.* Stanley and *E. coli* in regulation of proinflammatory cytokine expression. Cytokine TNF-α, IL-6 and IL-1β expression differed among serovars and bacterial conditions (live *vs.* killed). All the live bacteria inhibited TNF-α and IL-6 synthesis much more strongly than their heat-killed counterparts did; except live *S.* Typhi increased TNF-α release than the dead bacteria (Table 3). Interesingly, *S.* Stanley did not change the IL-1β expression between dead and live bacteria, compared that all live *Salmonella* bacteria increased the release of IL-1β. Comparison between *S.* Typhimurium and *S.* Stanley in cytokine secretion, *S.* Typhimurium stimulated significantly

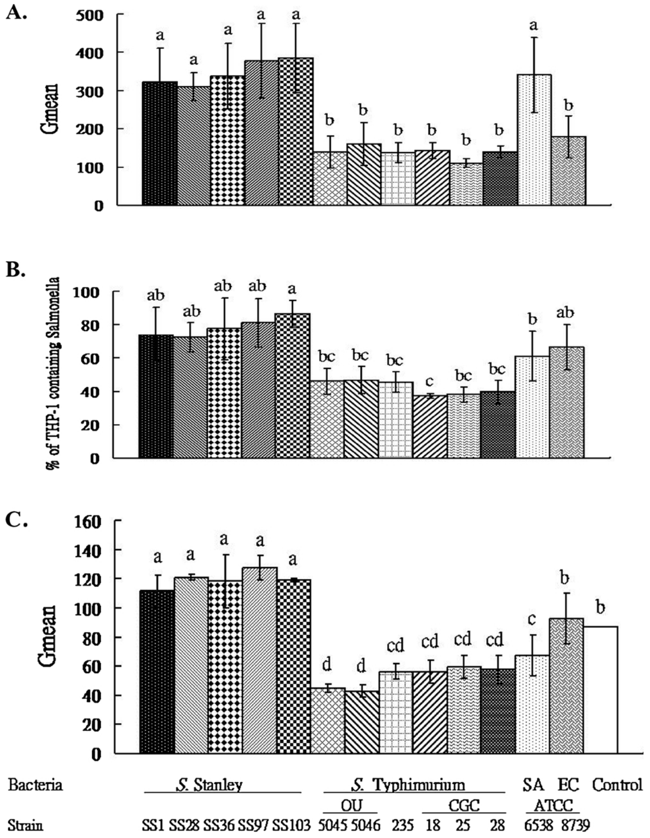


Fig. 3. The fluorescence intensity of DioC18-labeled *Salmonella* Typhimurium and *S.* Stanley in 1 × 10^4 THP-1 cells (A), the percentage of 1 × 10^4 THP-1 cells containing labeled bacteria (B), and reactive oxygen species (ROS) production by 1 × 10^4 PI-negative THP-1 cells infected by *S.* Typhimurium or *S.* Stanley (C). Different letters represent significant differences between the isolates ($p < 0.05$). The value was calculated by 3 independent experiment each with two repeats.

more TNF-α expression than *S.* Stanley. On the contrary, dead *S.* Stanley stimulated far more IL-1β expression than the live and dead *S.* Typhimurium.

4. Discussion

Salmomnella infection in human decreased from 10,730 during the period 2004 – 2007 to 7544 during 2008 – 2012; on the contrast, the total number of serotypes increased from 80 to 92 (Table 4). The top 10 prevalent serovars accounted for over 80% of *Salmonella* infections, and did not alter mostly during these two periods. Based on PFGE analysis, these top 10 prevalent serovars were separated into a genetically diverse group, such as *S.* Typhimurium, and a genetically conserved group, such as *S.* Enteritidis and *S.* Stanley. In contrast to the decrease in prevalence of other serovars from 2004 – 2007 to 2008 – 2012, only *S.* Enteritidis and *S.* Typhimurium increased in prevalence. Further, the common PFGE pulsotype ratio differed in three patterns among 9 serovars: 1) signifcant ratio increase for *S.* Stanley, *S.* Paratyphi B var. Java and *S.* Weltevreden, 2) ratio increase for S. Enteritidis, *S.* Newport, *S.* Albany, *S.* Derby, and 3) ratio decrease for *S.* Typhimurium and *S.* Agony (Table 5).

These data demonstrate that most serovars may adapt to human by some major clones, especially for S. Stanley, except *S.* Typhimurium and S. Agony evolve differently by increase in genotypes over two periods, possibly due to their higher mutation and/pr genetic exchange.

Clonally disseminated *S.* Stanley has been reported in the isolates from aquatic products [30] and beef, pork and buffalo meat [31]. In Egypt, major serovars from domestic and imported duckling include *S.* Enteritidis, *S.* Typhimurium, and *S.* Virchow, whereas *S.* Kentucky, *S.* Stanley, and *S.* Anatum are isolated from imported ducklings [32]. In Czech Republic, *S.* Stanley is one of the major serovar found in 22 outbreaks [33]. Within serogroup B, *S.* Typhimurium and *S.* Stanley differed in prevalence, host range, and invasiveness. *S.* Stanley frequently causes outbreaks through consumption of contaminated foods, such as peanuts [34], cheese [35], and turkey [36]. Such outbreaks are often associated with clonal dissemination (Fig. 1) [9,36].

In the present study, *S.* Stanley is clonal dissemination of a major pulsotype 2 with resistance to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole. The resistance rate to these above four drugs differed by regions, with a prevalence of more than 70% in Taiwan (Fig. 1) [37] and Thailand [38], compared to less than 10% in Hong Kong (9) and the Guangdong province of China [39]; and by sample sources, with lower than 50% to sulfafurazole (48.9%), followed by streptomycin (39.8%), tetracycline (25%), and nalidixic acid (21.6%) for strains from aquatic products [30], higher than 60% to streptomycin, tetracycline and ampicillin for the strains from market meals [40]. MDR S. Stanley s from cattle and bovine species were resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, oxytetracycline, and kanamycin in southern Japan [41,42]. Despite no ESBL strains detected, we identified the ESBL-realted genes *bla*$_{\text{TEM}}$ and *bla*$_{\text{CMY-2}}$ in the strains with resistance to β-lactam antibiotics, indicating the possible emergence of ESBL-producing *S.* Stanley.

Genome rearrangement, mobile element transfer and gene mutations can increase bacterial genome plasticity that may change bacterial pathogenicity and virulence [43]. In combination with host range, the evolutionary lineages may contribute to their prevalence: an increase in genome plasticity for *S.* Typhimurium, and a genome conservation for *S.* Stanley. Other than different genomic patterns, these two serovars to infect monocyte-like THP-1 cells differed in intracellular survival pattern, with a rapid decrease in intracellular *S.* Stanley and very little change in intracellular *S.* Typhimurium at early stage from 0 to 2 h (Fig. 2). Further, THP-1 cells response to *S.* Stanley than *S.* Typhimurium differed in two parts: more S. Stanley in each cells and more cells with S. Stanley than the S. Typhimurium (Gmean < 300,

Table 3
The expression levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-1β in CD14$^+$ monocytes incubated with different heat-killed or alive serovars.

Cytokine (pg/ml)	Bacterial condition	NC	*E. coli* pir116	*S.* Typhi OU7049	*S.* Stanley SS97	*S.* Typhimurium OU5046	*S.* Enteritidis OU7130
TNF-α	killed	0 ± 1.68^{a}	568.0 ± 53.03^{bc,y}	548.7 ± 60.56^{bc,x}	451.3 ± 41.30^{b,y}	732.4 ± 182.33^{c,y}	333.8 ± 101.81^{b,y}
	alive	0 ± 1.39^{a}	231.1 ± 22.30^{d,x}	828.8 ± 81.05^{e,y}	26.1 ± 2.90^{b,x}	101.1 ± 15.66^{c,x}	86.1 ± 4.35^{b,x}
IL-6	killed	0 ± 0.44^{a}	323.4 ± 49.70^{c,y}	257.2 ± 20.80^{bc,y}	226.8 ± 34.61^{b,y}	163.3 ± 73.79^{b,y}	163.1 ± 5.57^{b,y}
	alive	0.3 ± 1.66^{a}	34.8 ± 11.98^{b,x}	21.2 ± 3.32^{b,x}	4.2 ± 4.22^{a,x}	7.1 ± 2.36^{a,x}	6.3 ± 2.76^{a,x}
IL-1β	killed	1.0 ± 2.12^{a}	191.6 ± 60.50^{c,x}	49.6 ± 10.20^{b,x}	319.9 ± 29.98^{d}	164.6 ± 28.56^{c,x}	62.2 ± 32.01^{b,x}
	alive	8.4 ± 0.26^{a}	1208.8 ± 0.0^{c,y}	367.9 ± 13.35^{b,y}	363.8 ± 8.45^{b}	350.3 ± 10.82^{b,y}	363.0 ± 5.78^{b,y}

Each value was calculated by 3 independent experiments each with 3 repeats.
$^{a-e}$ indicates significant difference ($p < 0.05$) among bacterial groups.
^{x,y}indicates significant difference ($p < 0.05$) between killed and alive groups.

Table 4
Distribution of major serotypes during different periods.

2004-2007			2008-2012			2004-2012				
Serotypes	Serogroup	Total isolates [N, (%)]	Serotypes	Serogroup	Total isolates [N. (%)]	Serotype	Serogroup	Total isolates [N. (%)]	Total PFGE number	No. isolates/ PFGE pattern
Enteritidis	D	2786 (25.95)	Enteritidis	D	2339 (31.00)	Enteritidis	D	5125 (28.03)	193	26.6
Typhimurium	B	2340 (21.79)	Typhimurium	B	1846 (24.47)	Typhimurium	B	4186 (22.90)	678	6.2
Stanley	B	1048 (9.76)	Newport	C2	553 (7.33)	Stanley	B	1529 (8.36)	113	13.5
Newport	C2	701 (6.53)	Stanley	B	481 (6.38)	Newport	C2	1254 (6.86)	228	5.5
Albany	C2	445 (4.14)	Albany	C2	277 (3.67)	Albany	C2	722 (3.95)	171	4.2
Agona	B	432 (4.02)	Paratyphi B var. Java	B	221 (2.93)	Agona	B	645 (3.53)	94	6.9
Paratyphi B var. Java	B	326 (3.04)	Agona	B	213 (2.82)	Paratyphi B var. Java	B	547 (2.99)	113	4.8
Weltevreden	E1	310 (2.89)	Weltevreden	E1	195 (2.58)	Weltevreden	E1	505 (2.76)	212	2.4
Derby	B	296 (2.76)	Hadar/Istanbul	C2	195 (2.58)	Derby	B	452 (2.47)	98	4.6
Braenderup	C1	268 (2.50)	Derby	B	156 (2.07)	Bareilly	C1	365 (2.00)	32	11.4
Other 70 serotypes		1786 (16.63)	Other 61 serotypes		1068 (14.16)	Other 82 serotypes		2952 (16.15)	827	3.6
Total		10738 (100.00)	Total		7544 (100.00)	Total		18282 (100.00)	2759	6.6

Table 5
Genotype distribution of major serotypes during two periods.

Serovar	No. common PFGE patterns	Ratio (%) of number of common PFGE patterns to the total number of the PFGE patterns		
		2004-2007	2008-2012	Probability
Enteritidis	38	29.2 (38/130)	37.6 (38/101)	˜ 0.15
Typhimurium	93	25.3 (93/367)	23.0 (93/404)	˜ 0.30
Stanley	19	22.4 (19/85)	40.4 (19/47)	˜ 0.004
Newport	55	37.2 (55/148)	40.7 (55/135)	˜ 0.26
Albany	36	34.6 (36/104)	35.0 (36/103)	˜ 0.26
Agona	18	32.7 (18/55)	31.6 (18/57)	˜ 0.44
Paratyphi B var. Java	25	30.5 (25/82)	44.6 (25/56)	˜ 0.041
Weltevreden	34	22.2 (34/153)	36.6 (34/93)	˜ 0.01
Derby	24	36.4 (24/66)	42.9 (24/56)	˜ 0. 23

70 − 90% *vs.* Gmean < 200, 30 − 40%), howevedr, less intracellular bacteria for *S*. Stanley (Figs. 2, 3A and B). Furthermore, *S*. Typhimurium inhibited ROS production by THP-1 cells than *S*. Stanley did (Fig. 3C). These data imply that *S*. Stanley can stimulate THP-1cells to phagocytize them and produce more ROS that may play a major role in the early killing of intracellular *S*. Stanley.

Salmonella can stimulate type-1 inflammatory macrophages to produce the pro-inflammatory cytokines IL-23, IL-1β, and IL-18 and increase IL-23 production from monocytes in the presence of IFN-γ and granulocyte-macrophage colony-stimulating factor in response to a TLR agonist through the type-1 cytokine pathway [20]. The expression of TLR2 and CD14 by monocytes may affect the life span of patients with sepsis [44], and live *Salmonella* stimulated more IFNγ-producing γδ-T cells than heat-killed *Salmonella* did [45]. In the present study, we evaluated the expression of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 from human blood CD14$^+$ monocytes infected by different serovars at live and killed conditions (Table 3). The expression of IL-6 was lower and expression of IL-1β higher in the presence of live bacteria, indicating that active compounds, not surface structure, are involved in the inhibition of IL-6 expression; however, IL-1β expression is lipopolysaccharide-mediated and related to inflammasome formation. An early study reported that bacterial infection increased the serum concentrations of IL-6 and TNF-α than viral infections [46]. However, the live serovar *S*. Typhi induced higher TNF-α expression than dead bacteria with reverse phenomenon for other serovars, indicating that TNF-α expression may be important cytokine for infection by human-specific *S*. Typhi. Compared to *S*. Typhimurium, dead *S*. Stanley stimulated more IL-1β expression to enhance inflammation and may induce more rapid phagocytosis to eliminate *S*. Stanley than *S*. Typhimurium in early human infection.

5. Conclusion

Genetically diverse *S*. Typhimurium and clonally disseminated *S*. Stanley differed in immune response with higher phagocytized number, lower intracellular survival, higher ROS production and higher inflammation cytokine IL-1β expression by dead bacteria for *S*. Stanley, that may lead low prevalence of *S*. Stanley.

Conflict of interest

All authors declare no competing interests.

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