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Behavior variability of *Salmonella enterica* isolates from meat-related sources

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ABSTRACT

Salmonella enterica has been concerned globally due to its serious contamination in the food industry and serious risks to human health. In this study, the diversity of growth, genotypic virulence and clustered regularly interspaced short palindromic repeats (CRISPR) locus of 17 *Salmonella enterica* isolates from meat-related sources was evaluated. The results showed that the tested isolates exhibited significant differences ($p < 0.05$) in the growth kinetic behaviors, the growth rate was independent on serotype, and the fast-growing pattern was observed in Sinstorf serotype. The fold-change of nine genes involved in six virulence units showed isolate-to-isolate variation ($p < 0.05$), virulence genes associated with SPI-2 and SPI-4 displayed high expression. Relative lower expression levels of tested genes were obviously found in serotypes of Sinstorf, Stanley and Aberdeen. A considerable high diversity in repeats length and space numbers of both CRISPR-1 and CRISPR-2 among tested isolates was also observed, even within serotypes, which was also confirmed by the phylogenetic relationships. No significant relationship between the expression levels of each tested gene and the numbers of CRISPR spaces was observed. The information indicated that the variability among isolates of *Salmonella* with respect to their behavioral aspects is extensive, and assessment of strain variability is quite indispensable.

1. Introduction

Salmonella is recognized as one of the globally widespread food-borne pathogens. Although the total number of *Salmonella* outbreaks within the EU decreased markedly by 38.1% from 2008 to 2013, *Salmonella* was still the most frequently detected causative agent in the food-borne outbreaks reported in the EU (22.5%, EFSA-ECDC, 2015) and in the US (15.29%, CDC, 2016). Human acute gastroenteritis characterized by emesis and diarrhea was commonly linked to *Salmonella* infection associated with over 2500 serotypes. Most serovars preferred variety of habitats including humans and animals, disease cases associated with serovar/host combination (e.g. Typhimurium and child) have been confirmed in hospital (Krumkamp et al., 2016). Typhimurium and Enteritidis were the global predominant serotypes of *Salmonella* confirmed in outbreaks and food recalls. Most serotypes (except Typhimurium and Enteritidis) responsible for major outbreaks from Europe belonged to Hadar and Infantis, whereas most serotypes from North America belonged to Newport and Heidelberg (Vieira et al., 2009).

Salmonella infection is usually caused by cells invasion, which is closely related to virulence units including pathogencity island, plasmids, lipopolysaccharides and fimbriae. Each virulence unit includes many virulence genes, such as *invA* and *hilA* in type III secretion system of pathogencity island, *spvR* in plasmid and *fimA* in fimbriae. Given that genes encoding virulence ability evolved and varied at a high rate due to environment pressure of habitats even for the same strain, studies have demonstrated that the diversity of *Salmonella* strains or serotypes played a critical role in the virulence of cells (Shah, Zhou, Addwebi, Davis, & Call, 2011; Yim et al., 2010). McWhorter, Davos, and Chousalkar (2015) recently found that the *in vitro* invasive capacity of 17 *Salmonella* strains, determined using the gentamicin protection assay with the human intestinal epithelial cell line Caco-2, varied greatly with serotypes, and the survival percent of mice infected with Typhimurium was significant less than that of all other mice infected with Infantis, Lille, Montevideo and Oranienburg. The global transcriptome differences of six *S.* Enteritidis strains, previously characterized as high-pathogenicity and low-pathogenicity strains confirmed both

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in vitro and *in vivo* virulence assays, have also been revealed by Shah (2014) with RNA Sequencing. These studies suggested that it is essential to expand the knowledge of strain-to-strain or serotype-to-serotype variation in the virulence of food-associated *Salmonella* isolates.

Clustered regularly interspaced short palindromic repeats (CRISPR), which was first discovered in *Escherichia coli* in 1987, are present in about 50% of the sequenced bacteria including *Salmonella*. CRISPR region generally contains a newly discovered family of repeated DNA sequences characterized by direct repeats separated by variable sequences known as "spacers". Two CRISPR locus, which can provide insights into pathogen evolutionary, genetic diversity and transmission (e.g., vertical or horizontal) rates of a system, have been found in *Salmonella*. (Pettengill et al., 2014). Diversity of CRISPR locus have been explored as a subtyping tool within *Salmonella*, identifying strain differentiation at the serotype or sub-serotype level (Liu et al., 2011; Shariat, Sandt, DiMarzio, Barrangou, & Dudley, 2013b; Shariat et al., 2013a), and also could be used for tracking the farm-to-fork spread of the most prevalent serotypes of *Salmonella* during outbreak investigations. The direct or indirect relationships between CRISPRs and physiological characteristics of pathogens, such as virulence, attachment, commensalism, stress response and antibiotic resistance, have been recently revealed (Jiang, Yin, Dudley, & Cutter, 2015; Belkum et al., 2015; Louwen, Staals, Endtz, van Baarlen, & Oost, 2014; Zegans et al., 2009). It is quite essential to obtain more details insight into the diversity of CRISPR locus of *Salmonella*. The present study was therefore carried out to gain insights into the growth, virulence and CRISPR characteristics of meat-related *Salmonella* isolates, which would be of benefit for epidemiological investigations and microbial risk assessment in food.

2. Materials and methods

2.1. Strains and incubation medium

A standard *Salmonella* strain (ATCC 14028) and 16 isolates of *Salmonella enterica* collected from meat-related sources in 2014, were tested in this study. The details of isolates information is shown in Table 1. Stock cultures were maintained frozen at −70 °C in tryptone soy broth (TSB, Luqiao Technology Co. Ltd., Beijing, China) containing 40% glycerol. Prior to experiments, each isolate was activated twice on tryptone soy agar (TSA, Luqiao Technology Co. Ltd., Beijing, China) incubated at 37 °C for 24 h, and then each isolate was cultured in 10 mL TSB at 37 °C for 18 h. The cells were harvested by centrifugation at 12,000×*g* for 5 min at 4 °C and then washed three times with 0.85% NaCl solution.

2.2. Growth curves and kinetic parameters

The growth curves, expressed in terms of optical density of 600 nm, were determined as previously described (Diez-Garcia, Capita, & Alonso-Calleja, 2012). Growth curves were carried out in test tubes incubated in TSB at 37 °C. The initial concentration of each isolate was about 2.5 log CFU/mL confirmed by plate count method. The modified Gompertz equation was used to fit growth curves to the data of cells growth:

$$OD = A + (B-A)\exp\{-\exp[-\mu(t-M)]\}$$

$$\lambda = M-(1/\mu),\ \mu_{max} = (B-A)\,\mu/2.71828$$

Where OD_t was the optical density of 600 nm at time t; λ, the lag time in hours when the lag period ends; μ_{max}, the maximum growth rate achieved ($\Delta OD_{600\ nm}$/h); Values for A, B, μ, M, λ and μ_{max} were obtained by fitting a sigmoidal curve to the data set using SAS V8 software. Four repetitions were performed for each isolate.

2.3. Quantification expression of virulence genes

Total RNA of each isolate was isolated using an RNAiso plus Kit (Takara Bio, Dalian, China) according to the manufacturer's instructions, and then each RNA sample was treated with recombinant DNase I (Takara Bio, Dalian, China). The DNase-treated RNA was reverse-transcribed using the primescript RT master mix kit (Takara, 15 min at 37 °C). After dilution of cDNA, 2 μL of cDNA was used as a template for real-time PCR with random primers (Table 2) of nine virulence genes. Real-time PCR reactions were prepared as follows (10 μL final volume per sample): 5 μL of 2 × SYBR premix Ex SYBR (Takara Bio, Dalian, China), 0.2 μL of each primer (10 mM), 0.2 μL of ROX reference dyeII, 1.0 μL of cDNA template, and 3.4 μL of RNase-free water. A PCR system (QuantStudio 6 Flex, Applied Biosystems, Foster City, CA, USA) was programmed as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and a final melting curve program of 15 s at 95 °C and 1 min at 60 °C, followed with 15 s at 95 °C. In order to confirm that there was no background contamination, a negative control was included in each run, and 16s DNA was used as internal control since it is constitutively expressed under a wide range of conditions. The fold-change expression of target genes in comparison to 16s DNA were analyzed using QuantStudio™ 6 Flex software from Applied Biosystems (Foster City, CA, USA).

The $2^{-\Delta\Delta Ct}$ method was used to determine the expression levels of each target gene in comparison to internal control of 16s DNA (Livak & Schmittgen, 2001), where $\Delta\Delta Ct = (C_{t,\ virulence\ gene}-C_{t,\ 16sDNA})_{tested\ strain}-(C_{t,\ virulence\ gene}-C_{t,\ 16sDNA})_{ATCC\ 14028}$. The relative

Table 1
Serotypes and sources of *Salmonella* strains.

No.	Serotype	Source	R^2 of growth curve	No.	Serotype	Source	R^2 of growth curve
ATCC 14028	Typhimurium	ATCC	0.9968	NCM 1425	Chester	Uncertain	0.9951
NCM 1401	Sinstorf	Pork	0.9981	NCM 1427	Infantis	Convey chain	0.9972
NCM 1402	Stanley	Pork	0.9923	NCM 1431	Indiana	Convey chain	0.9921
NCM 1403	Aberdeen	Pork	0.9970	NCM 1438	Typhimurium	Convey chain	0.9885
NCM 1408	Derby	Pork	0.9983	NCM 1440	Weston	Chicken carcass	0.9983
NCM 1409	Agona	Pork	0.9965	NCM 1441	Rostock	Uncertain	0.9957
NCM 1415	Wandsworth	Pork	0.9950	NCM 1451	Enteritidis	Chicken breast meat	0.9989
NCM 1419	Aberdeen	Pork	0.9985	NCM 1456	Hadar	Chicken breast meat	0.9972
NCM 1422	Bury	Convey chain	0.9982				

NCM: National center of meat quality and safety control in China.
Uncertain: The strain was isolated from either pork or chicken meat.