Correlation between cluster analyses of *Salmonella* strains isolated from diarrhetic patients in Kuwait and biofilm formation

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Abstract

Salmonella is a highly diverse group of strains that belong to the *Enterobacteriaceae* and can cause many infections, such as diarrhea, pyrexia and septicemia, in humans and animals. One important virulence factor is the ability to form biofilm. In the present study, the potential to form biofilms by *Salmonella* strains isolated from diarrhetic patients was investigated and correlated with the strain type. Isolated bacteria were identified by sequencing of 16S rDNA. The potential of *Salmonella* to form biofilms was determined using bioluminescence microbial cell viability assay. In addition, the metabolic fingerprints of *Salmonella* were determined using the Biolog system, following the manufacturer's instructions. Cluster analysis based on catabolic activity and 16S rDNA of isolated strains showed the tendency of most Schwarzengrund (66.7% - 70%) and E5 strains (85%) to cluster individually, which implied the high distinctive genetic background of Schwarzengrund and E5 strains. On the other hand, Heidelberg and Paratyphi strains were clustered among other stains, which reflected the genetic resemblance of these strains to other *Salmonella* strains. The biofilm studies showed the high potential of the majority of E6 (60%) and Heidelberg (66.7%) strains to form biofilms, while low potential to form biofilms was displayed by 78% of Schwarzengrund strains.
Keywords: Salmonella, biofilm, 16S rDNA sequencing, metabolic fingerprint.

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1 Introduction

The enterobacteria are a heterogeneous group of Gram-negative rods that naturally inhabit the intestine of humans and animals. *Salmonella*, which is one of the important genera belonging to the *Enterobacteriaceae*, can cause many infections such as diarrhea, pyrexia and septicemia in humans and animals (D'Aoust [2]). *Salmonella* is usually found in poultry, poultry salads, meat, meat products, raw milk, shell eggs, egg custards, improperly cooked mayonnaise, ice cream, and sauces (Uyttendaele et al. [16]; Foley and Lynne [3]). Thus, *Salmonella* is one of the most common types of foodborne illnesses reported causing food poisoning or salmonellosis (D'Aoust [2]). Following the uptake of contaminated food or water, *Salmonellae* reach the intestine, cross the epithelial barrier, activate the host signal transduction cascades and induce the formation of membrane ruffles localized at the contact point between the bacterium and host cell (Jones et al. [5]; Vazquez-Torres et al. [17] and Rescigno et al. [12]). Ultimately *Salmonellae* are taken up in large vacuoles. The symptoms of salmonellosis develop within 12-36 hours of eating food containing *Salmonella*. Symptoms include nausea with vomiting, abdominal cramps and diarrhea, which can be severe (Tsolis et al. [8]; Kingsley et al. [8]; Ohl and Miller [11]). In recent years problems associated with *Salmonella* have increased considerably, both in terms of occurrence and severity of cases of human salmonellosis (Jewes [4]; Robertson et al. [14]).

An important factor influencing the pathogenicity of *Salmonella* is its ability to adhere to the host's intestinal surfaces prior to invasion (Robertson et al. [13]). This interaction is thought to depend upon bacterial-like adhesins recognizing specific glycoconjugate receptors on host cell surfaces. The possession of active flagella combined with chemotaxis is also an important factor in the pathogenicity of *Salmonellae* (Khoramian et al. [7]; Jones et al. [6]). Thus, variability in genotypic or phenotypic surface adhesion-related characteristics of different subspecies is expected to affect the establishment of *Salmonellae* populations in different hosts (Robertson et al. [13]) and thus its ability to cause outbreaks. One mechanism for ensuring survival in the host might be the differential biofilm-forming potential within a natural *Salmonellae* population. Biofilm formation is the net result of multiple interacting molecular events (Robertson et al. [13]) and is most conveniently measured at the phenotypic level. Thus, studies correlating strain types, antibiotic resistance and potential to form biofilm are required.

Many authorities require discrimination between different strains based on their metabolic pathways. Additionally, several high-resolution molecular fingerprinting techniques have been used to reveal species and subspecies diversity and provide tools to follow the persistence of particular infections, to recognize new infections and to assess the efficacy of control measures (Cheah et al. [1]). Typing methods based on comparisons of whole genomic DNA, plasmid DNA or specific genetic determinants have been used as supplementary techniques. Therefore, the aim of this work was to assess the ability of

Salmonella strains to form biofilm and the correlation of biofilm formation potential with the metabolic and the genetic background of the different strains.

2 Materials and methods

2.1 Sampling and initial identification of *Salmonella*

Salmonella strains were isolated from stool samples brought from diarrhetic patients to the public health laboratory, Ministry of Health. For the isolation of *Salmonella* from stools, stool samples were inoculated into selinite broth to enrich for *Salmonella*. After overnight incubation, loop-full from selinite broth were streaked onto Hektoen enteric agar and incubated at 37°C for 48 hours (Vernacchio et al. [18]). Suspected grown *Salmonella* colonies were inoculated into sterile Triple Sugar Iron (TSI) agar slants, incubated at 35°C for 24 hrs and *Salmonella* was identified following the Bacteriological Analytical Manual of the U.S. Food and Drug Administration (http://www.foodinfonet.com/publication/fdaBAM.htm). Identified *Salmonella* were stored in 15% glycerol nutrient broth at -40°C.

2.2 Molecular identification of *Salmonella* by 16S sequencing

Isolated bacteria were identified by sequencing of 16S rDNA. For this purpose, genomic DNA was purified from pure bacterial cultures using the Wizard Genomic DNA purification kit as recommended by the manufacturer (Promega). The concentration of extracted DNA were quantified by fluorometry with a model TK 100 fluorometer (Hoefer Scientific Instruments) by using the extended assay protocol of the manufacturer and then stored at -20°C. Then, 16S rDNA sequences were amplified from extracted DNA using 27F (AGAGTTTGATC(AC)TGGCTCAG) and 1492R (ACGG(CT)TACCTTGTTA CGACTT) primers (Kuske et al. [9]). All reactions were carried out in 25 µl volumes, containing 12.5 pmol of each primer, 200 µM of each deoxyribonucleoside triphosphate, 2.5 µl of 10x PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl; pH 8.3), and 0.5 U of *Taq* DNA polymerase (Applied Biosystems, UK), increased to 25 µl with sterile water. PCR was performed in a Thermocycler, GeneAmp (Applied Biosystems, UK) with the following thermocycling program: 5 minutes denaturation at 95°C, followed by 30 cycles of 1 minute denaturation at 95°C, 1 minute annealing at 55°C, 1 minute extension at 72°C, and a final extension step of 5 minutes at 72°C. PCR products were visualized by electrophoresis in 2% (wt/vol) agarose gels and with ethidium bromide (0.5 µg/ml) staining. Then, PCR products were used as templates for DNA sequencing reactions. The sequencing PCR conditions were the same as those described above. Amplified DNA was purified by using a QIAQUICK PCR cleanup kit (Qiagen, Inc.), and DNA concentrations were determined as mentioned previously. Approximately 100 ng of 16S rDNA will be used as a template in dye terminator cycle sequencing reactions (Applied Biosystems PRISM dye terminator cycle sequencing kit). The 16S rDNA sequences obtained

were run against the data bases using the basic alignment search tool (BLAST) and assigned to recognized representatives of the main eubacterial lineages based on scores of 97% or higher.

2.3 Biofilm formation by *Salmonella*

The potential of *Salmonella* for forming biofilms was determined using bioluminescence microbial cell viability Assay (Promega). For this purpose, 100 μL of overnight bacterial cultures (0.5 McFarland standard) were transferred to sterile polystyrene 96 well microplates followed by another 100 μL of phosphate buffer (pH 7). Microplates were incubated at 37˚C for 24 hrs. Then, plates were washed gently with sterile phosphate buffer; 100 μL of phosphate buffer was added followed by the addition of bioluminescence reagent. Contents of the plates were mixed on an orbital shake, incubated at room temperature for five minutes and luminescence values were taken on a microplate luminescence detector LD 400C (Beckman Coulter, USA). Control wells containing the phosphate buffer without cells were run to obtain a value for background luminescence.

2.4 Determination of metabolic fingerprinting of *Salmonella*

The metabolic fingerprints of *salmonella* were determined using a Biolog system following the manufacturer's instructions (OmniLog® ID System).

3 Results

Three different approaches were used to analyze and cluster isolated *Salmonella*. The phylogenetic analysis of the 16S sequences using 97% similarity index demonstrated the presence of five main phylotypes (Figure 1). Each phylotype was composed of different strain types. However, cluster analysis based on the ability of isolates to utilize different organic substrates showed the presence of highly diverse metabolic potentials of isolated *Salmonella* (Figure 2). Furthermore, isolated *Salmonella* were segregated into three main groups based on their potentials to form biofilm: isolates with high potential to form biofilm (Table 1(a)), isolates with low potential to form biofilm (Table 1(b)) and isolates unable to form biofilm (Table 1(c)). Each group contained different strain types.

4 Discussion

Cluster analysis based on the catabolic activity (Figure 2) and 16S rDNA (Figure 1) of isolated strains showed the tendency of most Schwarzengrund (66.7% - 70%) and E5 strains (85%) to clusters individually, which implied the high distinctive genetic background of Schwarzengrund and E5 strains. On the other hand, Heidelberg and Paratyphi strains were clustered among other strains, which reflected the genetic resemblance of these strains to other *Salmonella*

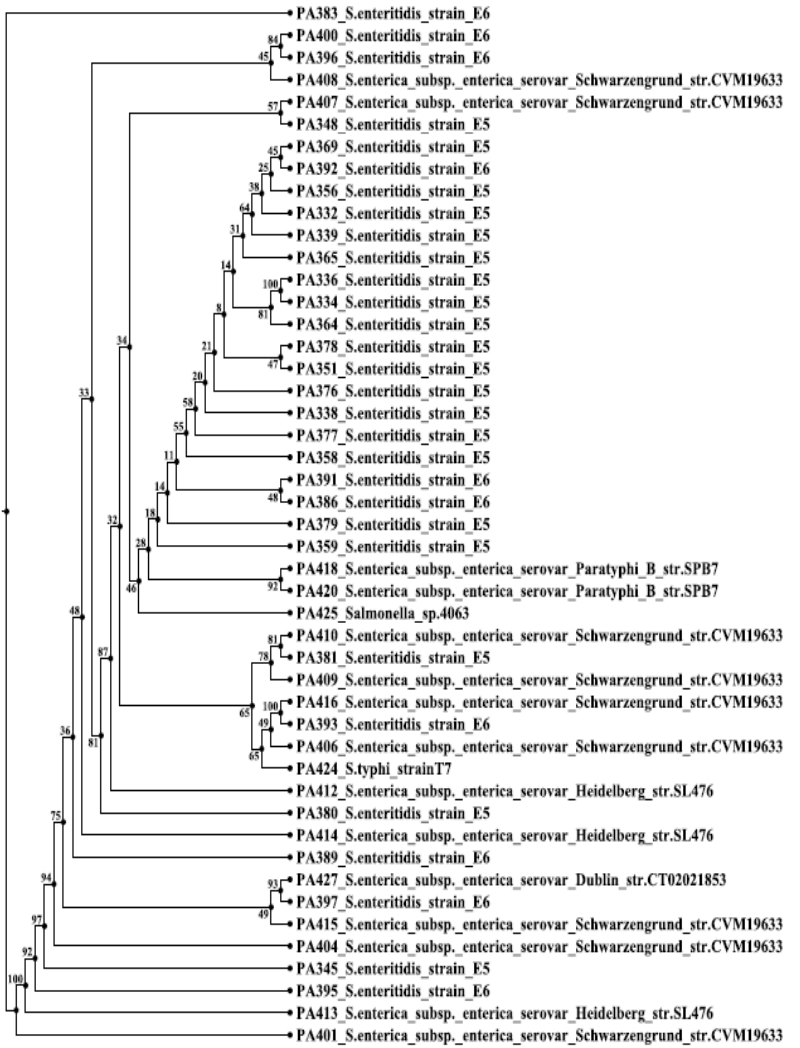

Figure 1: Phylogenetic tree of 16S rRNA gene sequences clustered using the UPGMA method. The numbers at the nodes represent percentages of bootstrap sampling.

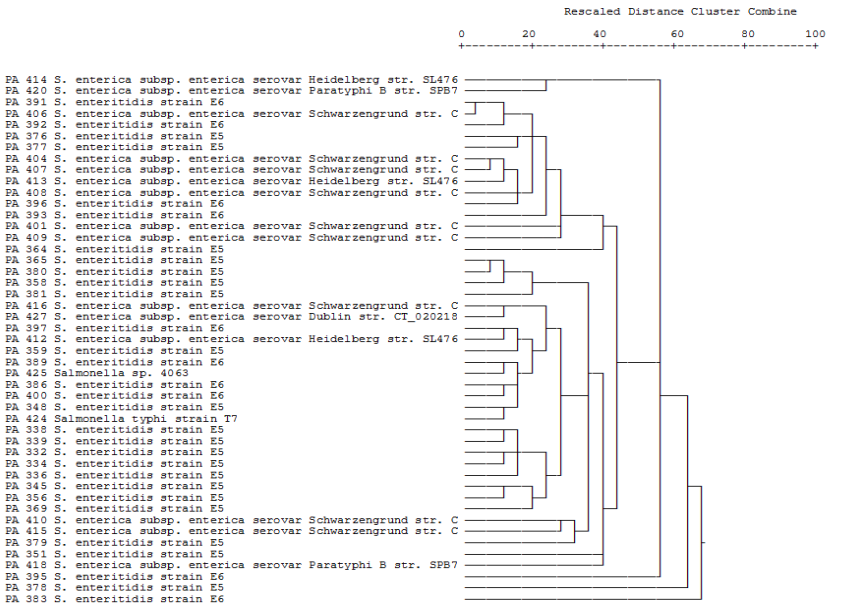

Figure 2: Cluster analysis tree of *Salmonella* based on metabolic fingerprinting obtained by Biolog.

Table 1: Biofilm formation by *Salmonella* strains. (a) *Salmonella* strains with high potential to form biofilm; (b) *Salmonella* strains with low potential to form biofilm; (c) *Salmonella* strains unable to form biofilm.

(a) *Salmonella* strains with high potential to form biofilm.

Sample No.	Strain identity
PA348	*S. enteritidis* strain E5
PA339	*S. enteritidis* strain E5
PA351	*S. enteritidis* strain E5
PA338	*S. enteritidis* strain E5
PA377	*S. enteritidis* strain E5
PA345	S. enteritidis strain E5
PA391	*S. enteritidis* strain E6
PA393	*S. enteritidis* strain E6
PA389	*S. enteritidis* strain E6
PA397	*S. enteritidis* strain E6
PA395	*S. enteritidis* strain E6
PA383	*S. enteritidis* strain E6
PA412	*S. enteric serovar Heidelberg*
PA413	*S. enteric serovar Heidelberg*
PA425	*Salmonella sp. 4063*

Table 1: Continued.

(b) *Salmonella* strains with low potential to form biofilm.

Sample No.	Name of bacteria from BLAST
PA369	*S. enteritidis* strain E5
PA336	*S. enteritidis* strain E5
PA334	*S. enteritidis* strain E5
PA364	*S. enteritidis* strain E5
PA358	*S. enteritidis* strain E5
PA379	*S. enteritidis* strain E5
PA400	*S. enteritidis* strain E6
PA396	*S. enteritidis* strain E6
PA392	*S. enteritidis* strain E6
PA415	*S. enteric* serovar Schwarzengrund
PA401	*S. enteric* serovar Schwarzengrund
PA407	*S. enteric serovar Schwarzengrund*
PA410	*S. enteric serovar Schwarzengrund*
PA418	*S. enteric serovar Paratyphi B*
PA420	*S. enteric serovar Paratyphi B*
PA424	*S. typhi strain T7*
PA414	*S. enteric serovar Heidelberg*
PA427	*S. enteric serovar Dublin*

(c) *Salmonella* strains unable to form biofilm.

Sample No.	Name of bacteria from BLAST
PA408	*S. enteric* serovar Schwarzengrund
PA409	*S. enteric* serovar Schwarzengrund
PA406	*S. enteric* serovar Schwarzengrund
PA404	*S. enteric* serovar Schwarzengrund
PA365	*S. enteritidis* strain E5
PA378	*S. enteritidis* strain E5

strains. The biofilm studies (Table 1) showed that the majority of E6 (60% - Table 1 (a)) and Heidelberg (66.7% - Table 1 (b)) strains were able to form biofilm with different potentials, while a very low potential to form biofilm was displayed by 78% of Schwarzengrund strains.

5 Conclusions

Some *Salmonella* strains demonstrated high potential to form biofilm while other strains showed low potential to form biofilm. Biofilm formation potential was not correlated with the metabolic or the genetic background of the tested strains.

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