Research Note

Survival of an Emerging Foodborne Pathogen - Group B *Streptococcus* (GBS) Serotype III Sequence Type (ST) 283 – under Simulated Partial Cooking and Gastric Fluid Conditions

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Short title (45 characters): GBS ST283 survival under heat and acid stress

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Abstract

Group B *Streptococcus* (GBS) was previously not known to be transmitted through food, but an outbreak investigation in Singapore in 2015 documented for the first time an association between GBS Type III Sequence Type 283 infection and consumption of raw fish dishes. As very little is known about the survival of GBS during heat treatment and the stomach transit, its survival under simulated conditions was studied, in comparison with that of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. The mean *D*-values of four GBS strains ranging from 0.72 to 0.88 min in neutral pH tryptone soy broth at 56.4 °C and 0.44 to 1.43 min at pH 2.35 at 37 °C in simulated gastric fluid, were significantly lower (P <0.05) than those of *E. coli* O157:H7 and *L. monocytogenes*. This study suggests possible factors other than acid or heat resistance of GBS to be instrumental to its pathogenicity.

Keywords: Group B *Streptococcus,* foodborne GBS*,* emerging foodborne pathonge, heat resistance, acid resistance.

Introduction

Group B *Streptococcus* (GBS), also known as *Streptococcus agalactiae*, is an invasive human pathogen responsible for neonatal sepsis, meningitis and maternal sepsis while also being an opportunistic pathogen in immunocompromised adults and the elderly (Le Doare and Heath, 2013). Its significance as a veterinary pathogen capable of causing economic loss from high fish mortality in aquaculture is also well documented (Delannoy et al., 2013).

Despite being known as a human and veterinary pathogen, GBS was not previously widely considered to be a foodborne pathogen. One study established a link between frequent consumption of fish and increased risk of colonization by GBS serotypes Ia and Ib (Foxman et al., 2007). Although evidence of common GBS serotypes being isolated from both fish and humans was previously reported (Delannoy et al., 2013; Ye et al., 2011), there was still insufficient substantiation of GBS presenting a foodborne risk to humans until recently.

The significance of GBS as a foodborne pathogen has only been recognized recently due to a large unprecedented outbreak associated with raw fish consumption in Singapore in 2015 (Chau et al., 2017b). Severe symptoms reported in the outbreak included meningoencephalitis, bacteremia, and septic arthritis (Rajendram et al., 2016; Tan et al., 2017; Tan et al., 2016a). To our knowledge, these were the first few reports of definitive epidemiological link indicating evidence of GBS being transmitted foodborne.

As GBS is not widely recognized as a foodborne pathogen, little is known on its resistance to heat and acid stress, which could play an important role in its survival in

68 food and the human stomach, and thus in its foodborne transmission. In this exploratory
69 study, we evaluated the survivability of GBS serotype III ST283(a) – a representative
70 strain of the 2015 Singapore outbreak clone – under heat (56 ^{o}C) and acid stress (pH
71 2.35) conditions. The heat-stress condition was established based on prior knowledge that
72 some patrons preferred to dip the raw fish slices in warm porridge before consumption
73 whereas others consume the fish entirely raw (Chau et al., 2017b). The acid-stress
74 condition, on the other hand, was designed to mimic the gastric environment of a healthy
75 human host. The survival of GBS ST283(a) was then compared with that of three other
76 GBS serotype III strains, as well as with that of two other foodborne pathogens – *Listeria*
77 *monocytogenes* and *Escherichia coli* O157:H7, which are known for their remarkable
78 heat resistance (Doyle et al., 2001; Yuk and Marshall, 2003). The overall findings of this
79 study will serve to provide insights on the physiological traits of GBS from the
80 perspective of a foodborne pathogen, as well as to generate new hypotheses for further
81 understanding the epidemiology of GBS foodborne infection.
82
83 **Materials and Methods**
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85 **Bacterial strains and culture conditions**
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87 The strains used in this study are presented in Table 1. In addition to GBS
88 ST283(a), a representative strain that caused the Singapore's foodborne outbreak in 2015,
89 three other non-outbreak-related GBS strains: GBS ST283(b), GBS ST335 and GBS
90 ST651, were included in this study. Although GBS ST283(a) and (b) share the same

sequence type 283, GBS ST283(b) was shown to differ from the representative outbreak strain by 58 single-nucleotide polymorphisms (SNPs) and was not found to cluster with the human outbreak strains (Chau et al., 2017b).

Frozen cultures of the strains preserved on porous cryobeads at -70 °C were activated by transferring each strain bead into 10 ml of tryptone soya broth (TSB; Oxoid, Basingstoke, UK) followed by incubation at 37 °C for 24 h. The approximate concentration of the 24 h culture was 10^9 CFU/ml. After two consecutive transfers, 1 ml of each culture at stationary phase (20 – 24 h) was centrifuged at 8,000 × *g* at 4 °C for 5 min and washed twice with 0.1% (w/v) peptone water (PW; Oxoid). The stationary phase was chosen as bacteria are largely known to be more heat- and acid-tolerant then, than at exponential phase. The resultant pellet was re-suspended in 1 ml of TSB for heat treatment or 1 ml of 0.1% PW for acid treatment, followed by serial dilution to approximately 10^7 CFU/ml. A cocktail culture consisting of three strains of *E. coli* O157:H7 strains was prepared by mixing 333 µl of each strain at stationary phase (20 – 24 h) in TSB, centrifuged, washed and re-suspended in 1 ml of TSB for heat treatment or in 0.1% PW for acid treatment followed by serial dilution to approximately 10^7 CFU/ml. The exact concentration of the inoculum was determined by plate count method at t = 0s. A cocktail culture consisting of three *L. monocytogenes* strains was also prepared likewise. While GBS strains were analyzed individually for inter-GBS strain comparison, cocktail cultures of *E. coli* O157:H7 and *Listeria* were prepared respectively to produce representative heat and acid resistance data of the two bacteria species.

Determination of heat resistance

The determination of heat resistance was performed as prevoiusly described (Yang et al., 2014). A 0.1 ml portion of approximately 10^7 CFU/ml inoculum in TSB was added separately to five test tubes containing 9.9 ml of pre-warmed TSB in a thermostatically controlled water bath (Polyscience, Niles, IL, USA) set at 56 °C to simulate the approximate temperature of warm porridge sold at stall. The absolute temperature of the TSB was measured to be 56.4 °C and maintained during the experiment. The intervals for measuring the effect of heat treatment on GBS, *L. monocytogenes* and *E. coli* O157:H7 were every 20-30 s, 4 min, and 5 min, respectively. At each respective time interval, a test tube of each GBS strain and cocktail culture of *E. coli* O157:H7 and *L. monocytogenes* was taken out of the water bath and immediately immersed in ice water to stop the heat treatment. Bacterial count at each period of heat treatment was enumerated by the automatic colony counter (Acolyte, Synbiosis, Frederick, MD, USA) after serially diluting in TSB, spiral plating (WASP 2, Don Whitley Scientific Ltd., West Yorkshire, UK) onto tryptone soya agar (TSA; Oxoid) and incubation at 37 °C for 24 h for *E. coli* O157:H7 cocktail, 24 - 48 h for GBS strains and 48 h for *L. monocytogenes* cocktail. The initial population count at t = 0 min before heat treatment was determined by enumerating the inoculum used. The $\log_{10}$ viable cell count ($\log_{10}$ CFU/mL) was plotted against time in min to construct the survival curves. The best fit line of survival curves was determined by linear regression with Microsoft Excel (Microsoft Corp., Redmond, WA, USA). The *D*-values (min) of strains were calculated by taking the negative reciprocal of the slope of the best fit line. Decimal reduction time

denoted as *D*-value, refers to the time required for a 1 log reduction of bacterial population at a given temperature or at a given pH (ISO, 1994).

Determination of acid resistance

Determination of acid resistance was performed and simulated gastric fluid (SGF) was prepared as previously described (Yang et al., 2014), consisting of 8.3 g/L of proteose-peptone (Oxoid), 3.5 g/L of D-glucose (Goodrich Chemical Enterprise, Singapore), 2.05 g/L of NaCl (Goodrich Chemical Enterprise, Singapore), 0.6 g/L of KH_2PO_4 (Merck, Darmstadt, Germany), 0.11 g/L of $CaCl_2$ (Goodrich Chemical Enterprise), 0.37 g/L of KCl (Goodrich Chemical Enterprise), 0.1 g/L of lysozyme (Sigma, St Louis, MO, USA) and 13.3 mg/L of pepsin (Sigma). All the materials except for the enzymes, lysozyme and pepsin, were dissolved in deionized water and autoclaved. After filter sterilization (0.20-μm syringe filter), the enzymes were aseptically added and mixed with the other components. The final pH of the SGF was adjusted using 37% HCl (Sigma) to pH 2.35 ± 0.05.

In order to study the acid resistance of each strain, 1 ml portion of the approximately 10^7 CFU/ml bacterial inoculum was added into 49 ml of pre-warmed SGF at 37 °C in a thermostatically controlled water bath to simulate conditions in the human stomach. The time intervals for the acid treatment of GBS, *L. monocytogenes* and *E. coli* O157:H7 were every 10 s, 10 min and 1 h, respectively. The *D*-values (min) were determined as described previously. At appropriate time intervals, 1 ml of the inoculated

158 SGF was transferred into 9 ml of phosphate buffered saline (PBS) (Vivantis Inc., CA,
159 USA) to quench the acid treatment followed by enumeration as described previously.
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161 **Statistical analysis**
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163 The mean log values of bacterial counts for heat and acid treatment were obtained
164 from three independent trials with duplicate plating each unless otherwise stated. All data
165 were statistically analyzed by comparing the mean values using the unpaired t-test with a
166 significance level set at $P < 0.05$ using the web-based GraphPad Software
167 (https://www.graphpad.com/).
168
169 **Results and Discussion**
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171 The representative outbreak strain (GBS ST283(a)) was not found to survive
172 better than the other three GBS strains, or than *E. coli* O157:H7 and *L. monocytogenes*
173 cocktails under heat stress at a measured temperature of 56.4 °C (Fig. 1). Both GBS
174 serotype III ST283(a) and (b) were found to have the lowest $D_{56.4\,°C}$–value (0.72 ± 0.06
175 min) which implies that they were most vulnerable to sudden heat stress (Fig. 1B).
176 Though some variabilities were observed among the four GBS strains in response to heat,
177 their $D_{56.4\,°C}$–values were all significantly lower ($P < 0.05$) than those of *E. coli* O157:H7
178 and *L. monocytogenes* cocktails (Fig. 1A). This implies that GBS strains were much
179 weaker in heat tolerance than *E. coli* O157:H7 and *L. monocytogenes* at 56.4 °C.

For acid resistance, the $D_{pH\ 2.35}$–value of GBS serotype III ST283(a) was 0.32 ± 0.13 min, which was the lowest among the four GBS strains studied, though the value was not significantly different from that of GBS ST651 (Fig. 2B). The $D_{pH\ 2.35}$–values of all four GBS strains were significantly lower ($P < 0.05$) than that of *L. monocytogenes* cocktails; whereas the viable counts of *E. coli* O157:H7 remained stagnant at about log 5 CFU/ml for 250 min under pH 2.35 (Fig. 2A). This implies that GBS strains were much weaker in acid tolerance than *E. coli* O157:H7 and *L. monocytogenes* at pH 2.35.

Before the Singapore outbreak in 2015, GBS was more commonly known to cause invasive disease among neonates and vulnerable populations. The unusual mode of foodborne transmission of GBS documented in the 2015 outbreak has led to an interest to find out whether the outbreak could be triggered by any unusual heat and/or acid resistance of the organism. Thus, this study was conducted to determine the survivability of GBS serotype III ST283(a) in relation to three other GBS serotype III strains, as well as *E. coli* O157:H7 and *L. monocytogenes* under simulated partial cooking (56.4 °C) and gastric fluid (SGF, pH 2.35) conditions.

The findings of this study demonstrated that the representative outbreak strain GBS ST283(a) was not more heat- or acid-resistant than the other three non-outbreak-related GBS strains. All four GBS strains were found to be much more susceptible to heat- and acid- stress than *E. coli* O157:H7 and *L. monocytogenes,* as revealed by their significantly lower $D_{56.4\ °C}$- and $D_{pH\ 2.35}$–*values*.

It is well known that the ability of *L. monocytogenes* and *E. coli* O157:H7 to produce an array of heat shock proteins in response to sub-lethal heat stress could enhance heat resistance and is an instrumental component of virulence (Wesche et al.,

2009). The inferior heat resistance of the GBS strains may be due to the lack of physiological heat stress response mechanisms, unlike *L. monocytogenes* and *E. coli* O157:H7. The relatively low heat resistance of GBS may also possibly explain why only sporadic cases were observed among a larger population of patrons who consumed the dish from implicated stalls. As not all customers would have dipped the raw fish slices in warm porridge, those who consumed the fish entirely raw were more likely to be exposed to higher levels of GBS. Our findings revealed that 1-log reduction of GBS counts was achieved quickly in less than a minute of exposure at 56.4 °C. Within two minutes, the high starting population of GBS of more than 5 log CFU/ml had undergone at least 2 log reduction. Though the dipping of raw fish slices in warm porridge may have protected some consumers from GBS infection, it is important to note that other factors such as uneven distribution of GBS in food and the status of host immunity may have played a role in this outbreak. Even though the results of this study showed that mild heat treatment (56.4 °C) was sufficient to reduce GBS levels rapidly, the practice of partial cooking should not be encouraged as the infectious dose of GBS ST283(a) is still largely unknown. Further studies will be required to establish the infectious dose of GBS ST283(a) in humans, so as to help the industry and authorities set realistic tolerable limits for GBS levels in ready-to-eat food. Nevertheless, our results show that GBS ST283(a) is much more easily inactivated by heat in comparison to *E. coli* O157:H7 and *L. monocytogenes* and thus, its risk as a foodborne pathogen can be addressed by thorough cooking.

In the aspect of acid resistance, the $D_{pH\ 2.35}$-values of all four GBS strains were observed to be significantly lower than those of *L. monocytogenes* and *E. coli* O 157:H7.

The low acid resistance of GBS ST283(a) was rather unexpected for a foodborne pathogen, particularly because GBS has been reported to survive in other low pH environments in the human hosts, such as the anogenital mucosa and phagolysosomal compartment of macrophages (pH 3.5- pH 4.5) (Shabayek and Spellerberg, 2017). Other studies have also shown that GBS possesses acid-induced genes (*dpsA*, *glnQ* and *hvgA*) that help the organism in the areas of cell adhesion, host invasion and/or colonization (Samen et al., 2004; Santi et al., 2009; Tazi et al., 2010). It has also been described elsewhere that high acidity enhances the ability of GBS to form biofilm to enhance their chances of survival (Rosini and Margarit, 2015). As the pH of gastric fluid (pH 2.35) is much lower than the other acidic sites of the human hosts, the present results could perhaps be explained by GBS' possible lack of elaborate biochemical and regulatory mechanisms that enable the survival of *E. coli* O157:H7 and *L. monocytogenes* in extremely low pH gastric environment (Foster, 2004; Gandhi and Chikindas, 2007). The poor survivability of GBS under gastric acid stress has also led to the hypothesis that a relatively high infectious dose may be required to cause foodborne disease. This hypothesis is taking reference from the characteristics of another acid-sensitive foodborne pathogen - *Vibrio cholerae.* The acquisition of cholera, for instance, necessitates the ingestion of a large number of *V. cholerae* cells (>10^6 CFU) (Kothary and Babu, 2001), so that a few cells may survive to colonize the host's GI tract to cause infection (Foster, 2004). Host immunity factors such as achlorhydria condition or antacid treatment may also be a contributory factor as individuals with such conditions may not produce sufficient gastric fluid to inactivate bacteria.

To date, GBS ST283 is the only sequence type found to be associated with foodborne bacteremia disease (Chau et al., 2017b; Rajendram et al., 2016; Tan et al., 2016b), although both the representative outbreak strain (a) and the non-outbreak-related strain (b) of ST283 were found to be neither more heat nor acid resistant than the other sequence types tested in this study. This suggests that the ability to cause foodborne disease by GBS ST283(a) may not be attributable to its heat and acid resistance. Further studies are needed to understand the genetic and physiological basis behind the apparent superior virulence of GBS ST283(a) as compared to the other sequence types in causing outbreaks.

Our findings did not support the hypothesis that the unusual Singapore 2015 foodborne outbreak was due to heat- and acid- resistance of the causative organism. The representative outbreak strain, specifically GBS serotype ST283(a), was not found to survive better under heat- and acid- stress conditions than the three other non-outbreak-related GBS serotype III strains, as well as *E. coli* O157:H7 and *L. monocytogenes*. Though our findings have not identified the physiological determinant that supports the foodborne transmission of GBS, this study has pointed to the need for further investigation to establish the infection and virulence mechanisms of GBS through the foodborne route.

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Table 1. List of bacterial strains used in this study

Strain	**ID No. (Accession no)**	**Reference / Source**
GBS serotype III ST 283(a)*	SGEHI2015-95 (SAMN08111301)	Chau et al. (2017b)
GBS serotype III ST 283(b)	SGEHI2015-25 (SAMN08111300)	Chau et al. (2017b)
GBS serotype III ST 335	SGEHI2015-II33 (SAMN08107781)	Chau et al. (2017b)
GBS serotype III ST 651	SGEHI2015-IV45_2 (SAMN08107782)	Chau et al. (2017b)
L. monocytogenes serotype 1/2a	SGEHI2012-SSA83	Chau et al. (2017a)
L. monocytogenes serotype 1/2b	SGEHI2011-RI-S16	Chau et al. (2017a)
L. monocytogenes serotype 4b	SGEHI2011-SSA4-1	Chau et al. (2017a)
E. coli O157:H7	F12	Seattle
E. coli O157:H7	C7927	Apple cider (Food Safety Inspection Service)
E. coli O157:H7	ATCC 35150	Human feces (American Type Culture Collection)

* Representative strain of the clone that caused the 2015 GBS foodborne outbreak in Singapore