A study of different buffers to maximize viability of an oral *Shigella* vaccine.

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Abstract:

Live, whole cell killed and subunit vaccines are being developed for diarrheal diseases caused by *V. cholerae*, *Shigella* species, ETEC, and *Campylobacter*. Some of these vaccines can be administered orally since this route best mimics natural infection. Live vaccines administered orally have to be protected from the harsh acidic gastric environment. Milk and bicarbonate solutions have been administered to neutralize the stomach acid. For many *Shigella* vaccine trials, 100 to 120 ml of a bicarbonate solution is ingested followed by the live vaccine candidate, which is delivered in 30 ml of bicarbonate, water or saline. It is not clear if maximum bacterial viability is achieved under these conditions. Also, volumes of neutralizing buffer that are optimal for adults may be unsuitable for children and infants. To address these questions, we performed studies to determine the viability and stability of a *Shigella sonnei* vaccine candidate, WRSS1, in a mixture of different volumes of five different buffer solutions added to hydrochloric acid to simulate gastric acidity. Among the buffers tested, bicarbonate solution, rotavirus buffer and CeraVacx were better at neutralizing acid and maintaining the viability of WRSS1. Also, a much smaller volume of the neutralizing buffer was sufficient to counteract stomach acid while maintaining bacterial viability.

Keywords:

S. sonnei vaccine

WRSS1 vaccine

Acid neutralization buffer

Vaccine viability

Introduction

Enteric infections caused by bacterial pathogens are a major cause of diarrheal diseases in children in underdeveloped countries. Poor hygienic conditions aid the spread of the disease and so it is doubly important to vaccinate children to protect them. Live attenuated or inactivated vaccines are the most advanced in terms of vaccine development in the fight against enteric infections. Some of these candidates are in Phase I and 2b clinical trials. Live ETEC vaccine candidate ACE 527 [1], live attenuated *Shigella sonnei* vaccine WRSS1 [2, 3], and whole-cell killed *Shigella* or *Campylobacter* vaccine [4] are some examples. The vaccines are administered orally since this mimics the natural route of infection.

Oral vaccines have to be formulated and administered in a way that protects them from the harsh acidic gastric environment. For this purpose milk [5] or bicarbonate [6, 7] solutions have been administered to neutralize the stomach acid prior to the ingestion of oral vaccine candidates. Currently, in *Shigella* vaccine trials, 100-120 ml of the neutralizing buffer solution--usually sodium bicarbonate (2g in 150 ml of sterile water) -is administered, followed by the candidate vaccine in 30 ml water or saline. However, it is unclear if maximal bacterial viability is achieved under these conditions because little work has been done to evaluate the volume of buffer needed to neutralize a fasting volume of gastric acid while maintaining bacterial viability. In addition, suboptimal buffering has been shown to diminish immunogenicity of killed whole cell cholera vaccine [8].

Since children less than 5 years of age are the target for oral enteric bacterial vaccines in endemic countries, information on optimal volumes and buffer compositions to counterbalance stomach acidity will be invaluable to maximize viability of the ingested vaccine. This could lead

to more efficient colonization and higher rates of immune responses. The studies described here focus on evaluating a minimal volume of a given buffer that is required to neutralize one volume of gastric acid while maintaining a high bacterial viability of WRSS1. In this study, Simulated Gastric Fluid with a starting pH of 1.68 was used to represent gastric acid, and different buffers were evaluated to neutralize SGF while monitoring the viability of WRSS1. Some of the buffers had sucrose added to improve the taste of the buffer.

Materials and Methods

Simulated Gastric Fluid (SGF, Ricca Chemical Co., U.S.A.) is 0.7% v/v HCl containing 0.2% w/v sodium chloride.

Neutralizing buffers: The buffer solutions that were tested included bicarbonate solution (2 g of sodium bicarbonate in 150 ml sterile water), RotaVirus buffer (RVB, proprietary composition, described under US patent# 8,241,886 B2), CeraVacx solution (Cera Products Inc, Hilton Head Island, South Carolina) citrate-phosphate buffer, and phosphate buffer. Some of the buffers were also tested after the addition of 20% sucrose to improve the taste. Composition of buffers and their pH levels are listed in Table 1.

Vaccine strain and viability plating: Lyophilized vials of WRSS1 [9] that has previously been tested in U.S., Israeli, and Thai volunteers, are stored at -80°C. WRSS1 has maintained its viability and stability of its plasmid-containing Form I phenotype for over 15 years. After rehydration with 5 ml of sterile water for injection (SWI) the viability is 2×10^9 Colony Forming Units (CFU)/ml. Form I refers to the phenotype of the strain when the large virulence plasmid (VP) is present. Loss of the VP that occurs spontaneously changes the round, smooth colony phenotype of Form I colonies to large flat colonies with wavy uneven edges referred to as Form

II phenotype. Since WRSS1 is a live invasive vaccine candidate, the presence of the VP is critical for its activity. Since its manufacture WRSS1 has maintained a >90% Form I phenotype. A lyophilized vial of WRSS1 was removed from -80°C and allowed to warm up on ice for 30 minutes. The lyophilized material was gently rehydrated with 5 ml of SWI following a standard procedure, and the rehydrated vial was swirled gently for 30 seconds and allowed to incubate on ice for 15 minutes with intermittent gentle swirling to obtain a uniform suspension. One ml of the rehydrated vaccine was added to 9 ml of saline and 10-fold serial dilutions were carried out up to 10^{-7} . In order to calculate the viability, an appropriate volume of the vaccine at 10^{-5} , 10^{-6} and 10^{-7} dilutions were plated in triplicate on Tryptic Soy Agar (TSA) plates. The plates were incubated overnight for 12 ± 2 hours at 37°C. The dilution which yielded between 30 and 300 CFU was used for the calculation of viability, which was expressed as CFU/ml.

Acid neutralization and plating: SGF and buffer solution were mixed in ratios between 1:0.125 and 1:3 as shown in Table 1. The schematic of the neutralization and plating protocol is shown in Figure 1. In brief, 2.375 ml of the SGF-buffer mixtures were aliquoted into two sets of sterile 15 ml round-bottomed tubes with caps and placed in a 37°C water bath. One set was used for pH measurements before and after addition of WRSS1, and the other set was used for plating at 0, 30 and 60 minutes after the addition of the vaccine. For example, from the WRSS1 serial dilution tube of 10^{-5} , 0.125 ml was added to one of the tubes containing the SGF-buffer mixture at 37°C. The tube was mixed gently by vortexing and immediately 50 µl of it was plated on TSA in triplicate. This would constitute T=0 for this mixture at this pH. The tube was placed back at 37°C and the plating repeated after 30 and 60 minutes. The plates were incubated at 37°C for 12 ± 2 h. As a control, 0.125 ml of vaccine from the same dilution tube was added to 2.375 ml of saline at 37°C and 50 µl was plated at the set time points. The number of colonies on each plate

was counted after the incubation period. The total number of colonies per ml of the SGF-buffer mix was calculated and compared to the total number of colonies per ml of saline. Results were expressed as % CFU/ml of saline control. Each experiment with an individual buffer was repeated three times and the average viability count was expressed as mean $\pm$ SEM.

Results and Discussion

Fecal shedding has been shown to be indicative of good immune response to oral vaccine administration [10, 11]. In order to be excreted as live bacteria in stool samples the bacteria must survive the harsh acidic environment of the stomach and colonize the distal colon. Although different buffers such as bicarbonate solution, bicarbonate-citrate buffer or milk have been used to neutralize gastric acid, it is unclear if the buffering is enough to maintain maximum viability of the live vaccine in the stomach, which turns over its contents into the intestine every 15-45 minutes based upon its constituents [12, 13]. The approximate acid output varies from 1.5 mEq/h in an infant weighing about 7.8 kg to 14 mEq/h in an adult weighing about 70 kg [14]. Titration experiments with 0.1 N HCl and antacid solution containing 1.6 g of sodium bicarbonate and 0.6 g of citric acid have shown that the age-specific doses of antacid given exceeded the amount of buffer required for neutralization of gastric acid, resulting in a lower immune response [8]. Hence it is important to administer an optimal amount of a neutralizing buffer to obtain a robust immune response.

The different buffers tested in this study had different neutralization potentials based on their starting pH and their buffering capacity (Table 1). Viability data from acid:buffer ratios which resulted in very low pH of the mixture and/or zero viability are not reported. Sodium bicarbonate solution at 2 g/150 ml water, considered equivalent to 1 volume of gastric acid, is usually administered to volunteers prior to ingestion of a live oral vaccine or challenge strain [15,

16]. The vaccine itself is administered in either 30 ml of bicarbonate solution, or in water, or in saline [17, 18]. But administering 100-120 ml of bicarbonate across all age groups is not ideal since the basal acid secretion varies from 3.3 ml/h in a day-old neonate to 64 ml/h in a 24-week infant, to 143 ml/h in an adult's stomach [14, 19].

In this study different volumes of bicarbonate solution were added to a fixed amount of SGF representing the resting or fasting state of stomach acid in an adult (Figure 2a). At 0 hour, 0.25 volume of bicarbonate solution was sufficient to neutralize 1 volume of SGF and increase its pH from pH 1.6 to pH 6.7. The viability of the vaccine in this mixture was 90% of the control mixture which contained only WRSS1 in saline. When 0.5 volume of bicarbonate was added to 1 volume of SGF, the viability of WRSS1 decreased with time. This is significant because the mean half-time of gastric emptying of liquid meal has been shown to be 22.0 ± 2.6 minutes [12]. The stomach contents empty after about 40 minutes of ingestion and it would be ideal to maintain the viability of the vaccine candidate in the gastric environment until it emptied out of the stomach.

Viability of WRSS1 was also tested in mixtures of SGF and bicarbonate containing 20% sucrose. Sucrose addition was considered to enhance the palatability of the vaccine solution for infants and children. As shown in Figure 2b, a 1:0.25 ratio of SGF to bicarbonate buffer containing 20% sucrose was best at maintaining the viability of WRSS1 at 100% for up to one hour. Higher volumes of sucrose-containing buffer resulted in a drop in viability to about 10% by one hour. It should be noted here that addition of sucrose would increase the osmolarity of the gastric contents that would slow gastric emptying. However, whether this would affect the viability of the vaccine at this stage is not known and will require further evaluation. Compared to adults, who have an estimated fasting stomach volume of ~40-80 ml of gastric fluid, children and

infants have lower volumes, in the range of ~20-30 ml). Based upon the age of the volunteer, administering 0.25 volumes of bicarbonate for 1 volume of stomach acid is recommended to retain maximum viability of the bacteria for at least one hour. Giving lower volumes of bicarbonate may alleviate the GI symptoms such as gas and a bloated feeling of discomfort that accompanies the ingestion of 100-150 ml of bicarbonate solution that is currently administered to volunteers during live vaccine trials [20].

A second neutralization buffer used in this study is the Rotavirus buffer (RVB, generous gift from PATH, EVI, Seattle, WA). This buffer is used to neutralize stomach acidity prior to administration of the rotavirus vaccine and contains among other constituents a buffer with high concentrations of sugar and Zn. When different volumes of RVB were added to a fixed volume of SGF prior to the addition of WRSS1, the viability of the vaccine was similar to that observed with the bicarbonate solution. A 1:0.25 ratio of SGF:RVB was sufficient to neutralize the acidity of the SGF while maintaining the viability of the vaccine at about 85% (Figure 3). When the ratio of RVB to SGF was increased to 1:1, the viability of WRSS1 in this mixture dropped within 30 minutes to 60% of that at 0 hour. This was in contrast to the 100% viability seen at a SGF:buffer ratio of 1: 0.25 up to 1 hour. Rotavirus buffer with added sucrose was not tested. Other buffers tested included 0.5M phosphate buffer pH 8.0, a citrate-phosphate buffer, both tested with and without sucrose, and CeraVax. The 0.5 M phosphate buffer-SGF mixture was quite stable and did not show pH changes with addition of the vaccine. Unlike bicarbonate solution and rotavirus buffer, as little as 0.125 volume of 0.5 M phosphate buffer was sufficient to neutralize 1 volume of SGF while maintaining the viability of WRSS1 at 80% of control value up to 1 hour after the addition of vaccine (Figure 4a). Higher volumes of the buffer reduced the

vaccine viability. With addition of 20% sucrose, viability of WRSS1 was maintained at 100% at SGF:phosphate buffer ratio of 1:0.125 up to 1 hour (Figure 4b).

Addition of the citrate-phosphate buffer to SGF at different ratios did not initially affect the viability of WRSS1, but within 30 minutes, the viability dropped to almost 50% of the initial value (Figure 5a). Addition of 20% sucrose did not improve the results (Figure 5b). This would suggest that neutralization of stomach fluid with a citrate-phosphate buffer may not be an optimal choice if the desired time to keep the vaccine active within the stomach contents is at least 30 minutes or more.

CeraVacx is a buffer specifically designed to neutralize gastric acidity during vaccine administration and was used during live oral cholera vaccine trials with vaccine candidate Peru-15 [21]. CeraVacx is composed of a defined form of rice syrup (long chain carbohydrates) with sodium bicarbonate and trisodium citrate. A SGF:CeraVacx ratio of 1:0.5 was the best in maintaining viability of WRSS1 over a 1 hour period (Figure 6). Other ratios of SGF:CeraVacx showed decreased viability over time. CeraVacx could be a good buffer to use for two reasons: the palatability or taste of the buffer, and the lower volume required compared to the 100-120 ml bicarbonate currently used to neutralize stomach acid. The carbohydrate in CeraVacx is thought to stimulate rapid absorption of the buffer solution in the intestine, thus concentrating the vaccine and leading to better immune responses.

Conclusion

Oral administration of live vaccines has been shown to be simple and effective as long as they are administered after neutralization of gastric acidity. Several different buffers have

been previously used for this purpose. For live *Shigella* vaccine trials a bicarbonate solution is routinely used prior to the ingestion of the vaccine candidate. The studies described here indicate that much lower volumes of the neutralizing buffer than usually recommended are sufficient to neutralize the acidity of the stomach contents and maintain the viability of the vaccine. Increasing the amount of neutralizing buffer results in a lower titer of the vaccine over time. This was true for all of the buffers tested in this study. Besides the bicarbonate solution, the rotavirus buffer appeared to provide good viability for the vaccine. Although currently 100-120 ml of bicarbonate is given during clinical trials with live *Shigella* vaccine candidates, these studies indicate that a much lower volume, perhaps between 10-20 ml of bicarbonate may be sufficient to neutralize a fasting volume of gastric fluid (~40-80 ml) in adults. This needs to be tested in a clinical trial. Addition of 20% sucrose to the buffers did not change the buffer properties. Thus, the choice of buffer and the volume administered to neutralize stomach acid are important factors to consider when setting up vaccine clinical trials. The results from this study shed light on the neutralizing capacity of a few different buffers. It remains to be seen if the results obtained here translate to volunteers in a clinical trial setting and whether lowering buffer volumes such as bicarbonate solution results in increased vaccine colonization and immunogenicity.

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Figure legends

Figure 1: Schematic of acid neutralization and plating of WRSS1.

125 μ l of WRSS1 from the 10^{-5} serial dilution tube was added to two tubes containing 2.375 ml of SGF- buffer mixture at 37°C. One tube was used to measure pH before and after addition of WRSS1 and the other was used to plate at 0, 30 and 60 minutes.

Figure 2: Viability of WRSS1 in SGF:bicarbonate mixture in ratios from 1:0.25 to 1:1.5.

WRSS1 was added to a mixture of SGF:sodium bicarbonate solution (a) or a mixture of SGF:sodium bicarbonate containing 20% sucrose (b) and plated on TSA at 0, 30, and 60 minutes. Colonies were counted after 12 h incubation at 37°C and expressed as % of control CFU/ml. Results shown are mean $\pm$ SEM of three experiments done on different days.

Figure 3: Viability of WRSS1 in SGF:RVB in ratios from 1:0.25 to 1:1.

WRSS1 was added to a mixture of SGF:RVB and plated on TSA at 0, 30, and 60 minutes. Colonies were counted after 12 h incubation at 37°C and expressed as % of control CFU/ml. Results shown are mean $\pm$ SEM of three experiments done on different days.

Figure 4: Viability of WRSS1 in SGF:Phosphate buffer in ratios from 1:0.125 to 1:2.

WRSS1 was added to a mixture of SGF:0.5 M Sodium Phosphate buffer (a) or a mixture of SGF:0.5 M Sodium Phosphate buffer containing 20% sucrose (b) and plated on TSA at 0, 30, and 60 minutes. Colonies were counted after 12 h incubation at 37°C and expressed as % of control CFU/ml. Results shown are mean $\pm$ SEM of three experiments done on different days.

Figure 5: Viability of WRSS1 in SGF:Citrate-Phosphate buffer in ratios from 1:0.125 to 1:1.

WRSS1 was added to a mixture of SGF:Citrate-Phosphate buffer (a) or a mixture of SGF:Citrate-Phosphate buffer containing 20% sucrose (b) and plated on TSA at 0, 30, and 60 minutes. Colonies were counted after 12 h incubation at 37°C and expressed as % of control CFU/ml. Results shown are mean $\pm$ SEM of three experiments done on different days.

Figure 6: Viability of WRSS1 in SGF: CeraVacx in ratios from 1:0.5 to 1:3.

WRSS1 was added to a mixture of SGF: CeraVacx and plated on TSA at 0, 30, and 60 minutes. Colonies were counted after 12 h incubation at 37°C and expressed as % of control CFU/ml. Results shown are mean $\pm$ SEM of three experiments done on different days.

Table 1: Ratios of buffers used and their pH ranges.

Mixture	Acid:Base ratios tested	pH Range		Optimal acid:base ratio	Optimal pH
		Before vaccine addition	After vaccine addition		
SGF + HCO₃ solution	1:0.25 - 1:1.5	6.7 - 7.8	7.4 - 8.4	1:0.25	7.4
SGF + HCO₃ containing 20% sucrose	1:0.25 - 1:1.5	6.5 - 8.2	7.1 - 8.6	1:0.25	7.1
SGF + Rotavirus buffer	1:0.25 - 1:1	5.8 - 6.3	5.8 - 6.3	1:0.25	5.8
SGF + CeraVax	1: 0.5 – 1:3	6.7 - 8.3	7.0 – 8.5	1: 0.5	7.0
SGF + Citrate Phosphate buffer	1:0.125 - 1:1	4.7 - 6.3	4.7 - 6.3	1: 0.25	5.6
SGF + Citrate Phosphate buffer containing 20% sucrose	1:0.125 - 1:1	4.7 - 6.3	4.7 - 6.3	1: 0.25	5.6
SGF + Phosphate	1:0.125 - 1:2	6.7 - 7.7	6.7 - 7.7	1:0.125	6.7

buffer

SGF + Phosphate 1:0.125 - 1:2 6.7 - 7.7 6.7 - 7.7 1:0.125 6.7

buffer containing**20% sucrose**

Buffer Compositions:

Simulated gastric fluid – Ricca Chemical Co, Cat #7108-16 (starting pH 1.68).

HCO₃ solution – 1.33 g sodium bicarbonate in 100 ml sterile water (starting pH 8.33).

Rotavirus Buffer – Proprietary solution obtained from PATH, EVI (starting pH 6.3).

CeraVacx – 9.5 g CeraVacx powder in 200 ml sterile water (starting pH 9.3).

Phosphate buffer – 0.5 M sodium phosphate buffer (starting pH 8.0).

Citrate-Phosphate buffer – 0.026 M citric acid/0.114 M trisodium citrate dehydrate/ 0.112 M potassium phosphate dibasic (starting pH 6.5).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6