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Research paper

Invasive African nontyphoidal *Salmonella* requires high levels of complement for cell-free antibody-dependent killing

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ABSTRACT

Nontyphoidal isolates of *Salmonella* (NTS), particularly *Salmonella* Typhimurium, are a major cause of invasive bacteremia in Africa. Despite this, no vaccine against NTS is currently available for use in humans. If a NTS vaccine is to be developed in a timely manner, there is a need to develop assays to assess its *in vivo* efficacy. Assessment of potential efficacy of candidate vaccines in preclinical models is important for proof-of-concept and reduces attrition of vaccines in clinical trials. Serum bactericidal assays (SBA) are often used to assess the functional activity of vaccine-induced antibody responses targeted against Gram-negative bacteria with results given as the maximum dilution of serum that can effect bacterial killing. Previously we have found evidence for a protective role for antibody-induced complement-mediated killing of NTS in African children using an undiluted whole serum SBA. However, endogenous complement in diluted human sera is limiting and insufficient to effect bactericidal activity against *S.* Typhimurium beyond two two-fold dilutions. In the current study, we examined the requirements for SBA against NTS using baby rabbit serum (BRS) as an exogenous source of complement. We found that the amount of complement required for antibody-mediated bactericidal activity is much higher for the invasive African *S.* Typhimurium isolate D23580, compared with the laboratory *S.* Typhimurium LT2 and *Salmonella* Paratyphi A CVD1901. While 20% BRS was sufficient to kill LT2 and CVD1901, 75% BRS was needed to kill D23580. Our findings demonstrate that one concentration of exogenous complement is not suitable for SBA against all *Salmonella* isolates. To develop SBA to assess the *in vivo* efficacy of *Salmonella* vaccines, it is necessary to optimize the assay for the *Salmonella* isolates against which the vaccine is targeted.

1. Introduction

Salmonella enterica causes a spectrum of diseases, including typhoid and paratyphoid fever, and gastroenteritis (Everest et al., 2001; Hohmann, 2001; Boyle et al., 2007), and is a major threat to public health. *S. enterica* serovar Typhi is the causative agent of typhoid fever. Paratyphoid fever, a clinically-similar disease with less prevalence, is caused by *S. enterica* serovar Paratyphi A, B and C. In developed countries, nontyphoidal isolates of *Salmonella* (NTS) usually cause gastroenteritis. In Africa, NTS, especially *S. enterica* serovar Typhimurium, are a common cause of invasive disease, in particular bacteremia. NTS bacteremia in sub-Saharan Africa primarily occurs in children under 2 years of age and HIV-infected individuals (Graham et al., 2000; Graham, 2002, 2010; Brent et al., 2006; Bronzan et al., 2007). The estimated minimum incidence of NTS bacteremia is 175 per 100,000 in Kenyan

Abbreviations: NTS, nontyphoidal *Salmonella*; SBA, serum bactericidal assay; BRS, baby rabbit serum; MLST, multi-locus sequence typing; CFU, colony forming units; LB, Luria Bertani; LPS, lipopolysaccharide.

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children under 5 years of age per year (Berkley et al., 2005). The lack of specific clinical presentation of NTS bacteremia makes diagnosis difficult. In addition, increased drug resistance and the emergence of new multi-drug resistant isolates (Hohmann, 2001; Mirza et al., 1996) have added to the burden of this often fatal disease. These findings emphasize the need for an effective vaccine against NTS. Currently, none is available for use in humans.

The role of antibody in protection against *Salmonella* has been well established. Adoptive transfer of antibodies confers protection against virulent *Salmonella* challenge (Mastroeni et al., 1993; McSorley and Jenkins, 2000). The importance of antibodies has also been emphasized by studies on Vi polysaccharide, which elicit T cell-independent antibody production and confer protection (Acharya et al., 1987). A key assessment of most vaccines is their ability to induce specific antibody production. However, high antibody levels alone are insufficient, since vaccine-induced antibodies need to be protective. A common *in vitro* assay aimed at providing functional information about potential *in vivo* protection is the serum bactericidal assay (SBA). This assesses the complement-dependent bactericidal activity of antibodies in sera against particular bacterial isolates. SBA have been used to gauge natural immunoprotection against *Salmonella* in Africans (MacLennan et al., 2008; Pulickal et al., 2009), and are reported to be the best immunological surrogate of protection against meningococcal disease (Frasch et al., 2009). Using an undiluted whole human serum SBA, our previous data demonstrate the necessity of both antibody and complement for *in vitro* killing of *Salmonella* and provide evidence that bactericidal antibody protects against invasive NTS disease in Africans (MacLennan et al., 2008).

There are a number of variables associated with the design and optimization of SBA. Optimum conditions required for *Salmonella* SBA have not been reported. In the present study, we evaluated the complement requirements of SBA for three isolates of *Salmonella*: invasive African *Salmonella* Typhimurium D23580, laboratory *S.* Typhimurium LT2, and laboratory *Salmonella* Paratyphi A CVD1901, using both endogenous and exogenous complement.

2. Materials and methods

2.1. Study sera

Blood from healthy volunteers (1 European and 1 Asian) was allowed to clot and serum was separated within 2–3 h. Aliquots of sera (donor 1 and 2) were then stored at −80 °C to preserve complement function. Pooled Malawian serum was separated from blood samples taken from healthy adults in Blantyre, Malawi, and pooled prior to storage at −80 °C. All individuals had no known clinical history of *Salmonella* infection. Ethical approval was granted by the College of Medical Research and Ethics Committee, College of Medicine, University of Malawi.

2.2. Bacterial isolates

Three *Salmonella* isolates were used: *S.* Typhimurium D23580, *S.* Typhimurium LT2 and *S.* Paratyphi A CVD1901. *S.* Typhimurium D23580 is an invasive African isolate with MLST sequence type ST313 from a bacteremic child in Blantyre, Malawi (MacLennan et al., 2008; Kingsley et al., 2009). It is representative of most NTS isolates from bacteremic individuals in Malawi since 2002 and is sensitive to killing by healthy human adult serum (Kingsley et al., 2009; MacLennan et al., 2008). *S.* Typhimurium LT2 is a commonly-used laboratory isolate of *S.* Typhimurium (Hoiseth and Stocker, 1981). *S.* Paratyphi A CVD 1901 is a laboratory *guaA*$^{-}$ mutant from the Center for Vaccine Development, University of Maryland School for Medicine (Gat et al., 2011). Its attenuation permits the use of CVD 1901 in BSL2 containment laboratories. This isolate is unable to synthesize guanine. All bacterial isolates were grown aerated in 10 ml LB in loose-capped 50 ml Falcon tubes at 37 °C with shaking at 180 rpm.

2.3. Salmonella serum bactericidal assay

For serum bactericidal assays involving endogenous complement, 5 μl viable *Salmonellae* at 2 h log-growth phase and an OD of approximately 0.2 (with shaking at 180 rpm) was added to 45 μl undiluted or PBS-diluted serum (final *Salmonella* concentration 1×10^6 CFU/ml) and incubated at 37 °C with the number of viable *Salmonellae* determined by serial dilution on Luria Bertani (LB) agar after 0, 45, 90, and 180 min. For serum bactericidal assays with exogenous complement, 5 μl viable bacteria in log-growth phase was added to 45 μl of a mix of PBS-diluted heat-inactivated serum and baby rabbit serum (BRS). Test serum was heat-inactivated by incubating at 56 °C for 30 min. BRS were from AbD Serotec (Kidlington, UK) and Pel-Freez/Invitrogen (Milan, Italy).

2.4. Anti-Salmonella antibody assay

5 μl *Salmonellae* at 3 h log-growth phase was mixed with 45 μl 10% serum (final *Salmonella* concentration 2×10^8 CFU/ml) as previously described (MacLennan et al., 2008). Antibody bound to bacteria was detected with FITC-conjugated polyclonal goat anti-mouse IgG, IgA and IgM antibody (Sigma-Aldrich, Milan, Italy) prior to FACS analysis on a FACSCanto instrument (BD Biosciences, Milan, Italy).

2.5. LPS extraction

Overnight bacterial cultures were washed with 0.9% (w/v) NaCl and boiled in a solution of 60 mM Tris–HCl, 2% (v/v) SDS and 1 mM EDTA pH 6.8. RNase/DNase solution (Sigma-Aldrich) was then added at a final concentration of 100 μg/ml and incubated at 37 °C. Following this, proteinase K (Sigma-Aldrich) was added at a final concentration of 50 μg/ml. The LPS mixture was incubated overnight at 50 °C and then stored at 4 °C until use.

2.6. LPS gel electrophoresis and silver staining

Tris–acetate sample buffer (Invitrogen) was added to the extracted LPS. The mixture was then boiled and separated on a 16% Tricine gel (Invitrogen). After electrophoresis, the gel was fixed in 40% ethanol, 5% acetic acid for an hour before a 5 min incubation with an addition of 0.7% periodic acid. After three washes with distilled water, the gel was stained with

0.04 M $AgNO_3$, 0.013% (v/v) NH_4OH, and 0.0187 M NaOH and developed with 0.5% (v/v) citric acid and 0.05% (v/v) formaldehyde until the appropriate staining intensity was achieved. The reaction was terminated with 5% methanol (Tsai and Frasch, 1982).

3. Results

3.1. Diluted human serum has limited bactericidal activity against S. Typhimurium

All three *Salmonella* isolates used in the study, *S.* Typhimurium D23580, *S.* Typhimurium LT2 and *S.* Paratyphi A CVD1901, were morphologically smooth with long-chain lipopolysaccharide, as indicated by the characteristic ladder appearance of O-antigen repeating units of lipopolysaccharide visualized by SDS-PAGE with silver-staining (Fig. 1). This indicates that any susceptibility to serum killing is not due to the absence of the lipopolysaccharide O-antigen chain. We confirmed by flow cytometry that all sera used contained IgG, IgA and IgM against the three bacterial isolates. BRS did not contain any IgG, IgA and IgM against the isolates (Fig. 2).

We examined the bactericidal activity of diluted fresh human serum in SBA against the three *Salmonella* isolates. When used undiluted, all three human sera killed the isolates (where killing is defined as any reduction in viable bacterial count compared with the initial *Salmonella* concentration). More specifically, all three human sera killed *S.* Typhimurium D23580 by 2–3 $\log_{10}$ and *S.* Typhimurium LT2 by 3 $\log_{10}$ at 180 min, while *S.* Paratyphi A CVD1901 was killed by 3 $\log_{10}$ within 45 min (Fig. 3). Heat-inactivation (to remove complement activity) abolished serum bactericidal activity, consistent with bacterial killing being complement-dependent as previously shown for D23580 (MacLennan et al., 2008). *S.* Paratyphi A CVD1901 was highly sensitive to serum killing with all dilutions of human sera tested killing the bacteria. 1/2, 1/4, 1/8 dilutions effected a 3 $\log_{10}$ kill and 1/16 dilution a 1 $\log_{10}$ kill by 180 min. The bactericidal activities of the human sera against *S.* Typhimurium isolates were more affected by serum dilutions, particularly D23580 — the highest dilution of the human sera that could still kill LT2 was 1/8 for donor 1 and donor 2 sera, and 1/4 for the pooled Malawian serum, while 1/4, but not 1/8 dilution of all sera killed D23580. These findings indicate the limitation of

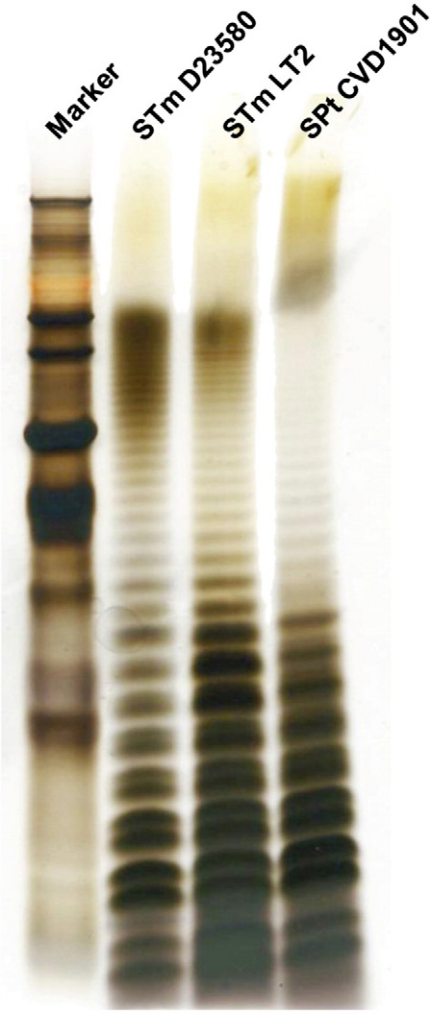

Fig. 1. LPS profiles of *Salmonella* isolates used in serum bactericidal assays (SBA). LPS was extracted from overnight cultures of *S.* Typhimurium D23580, *S.* Typhimurium LT2 and *S.* Paratyphi A CVD1901 and separated on a Tricine SDS-PAGE gel which was silver stained.

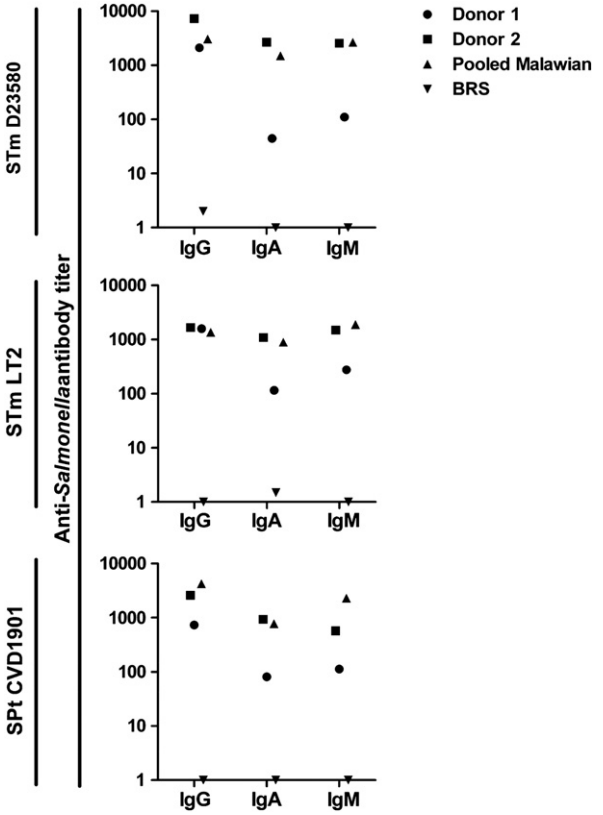

Fig. 2. Levels of anti-*Salmonella* antibody in human sera used in SBA. Antibody deposition on *S.* Typhimurium (STm) D23580, *S.* Typhimurium (STm) LT2 or *S.* Paratyphi A (SPt) CVD1901 in log-growth phase was measured by flow cytometry. Each point represents each of the 3 human sera or BRS.

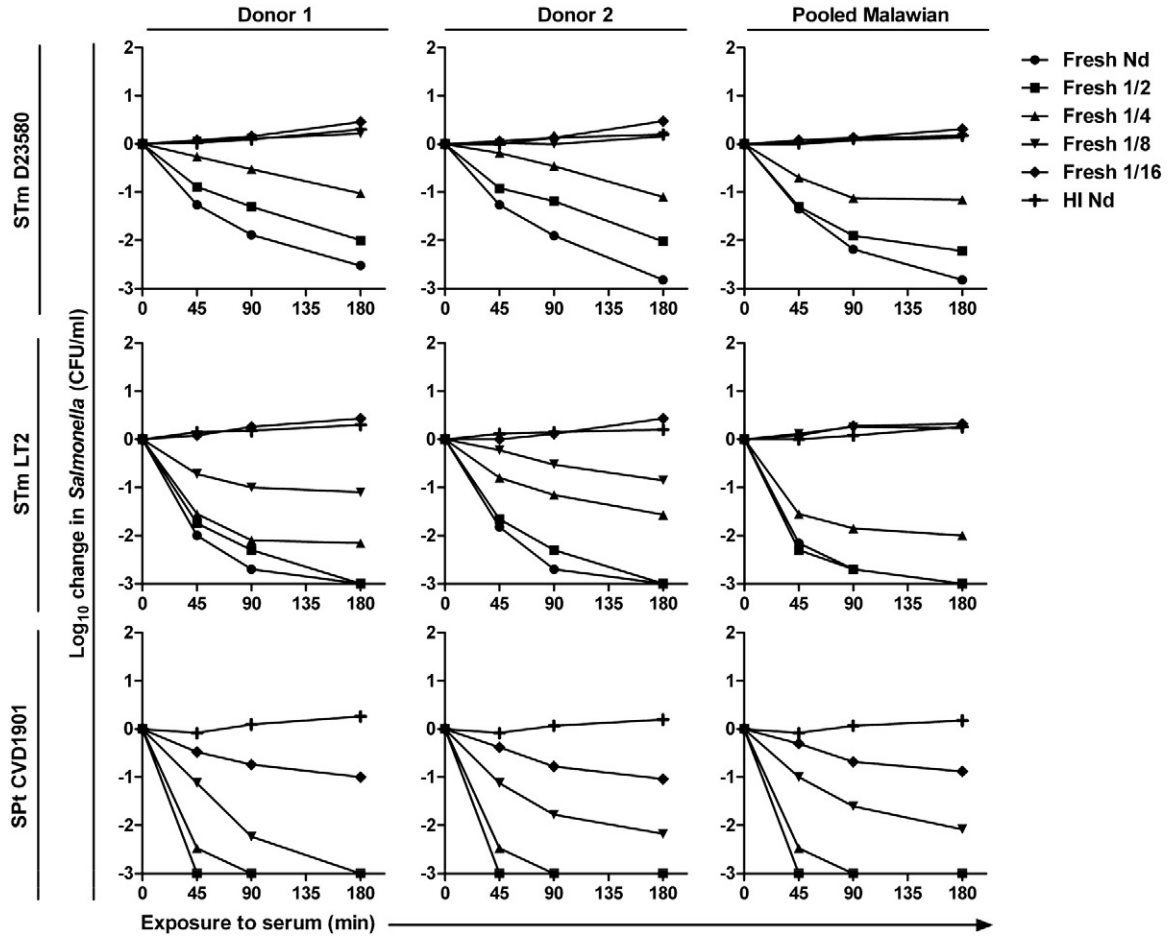

Fig. 3. Bactericidal activity against *Salmonella* isolates of human serum as source of antibody and complement. SBA was performed with 1×10^6 *Salmonella*/ml and the number of viable bacteria was determined following 45, 90, and 180 min of serum exposure. *S.* Typhimurium D23580, *S.* Typhimurium LT2 and *S.* Paratyphi A CVD 1901 were used as target isolates and three human sera (sera from donor 1, donor 2 and Malawian adults) were used as antibody source. The human sera were either non-diluted (Nd) or diluted (1/2, 1/4, 1/8, 1/16) in PBS. Heat-inactivation (HI) of human sera was performed by incubating the sera at 56 °C for 30 min.

using diluted human serum in serum bactericidal assays against *S.* Typhimurium. Since both antibody and complement are co-diluted, the individual contributions of anti-*Salmonella* antibody and complement to killing of *Salmonella* cannot be determined. Hence, it is necessary to provide an exogenous source of complement in *S.* Typhimurium serum bactericidal assay when serial dilutions of human serum are used as the source of antibody.

3.2. Fresh BRS is unable to kill S. Typhimurium in the absence of antibody

BRS is commonly used as an exogenous source of complement in serum bactericidal assays and was used as the exogenous source of complement in this study. We first measured the ability of BRS alone to kill *Salmonella* by determining the viable bacterial numbers following exposure to different percentages (20%, 50%, 75%, 100%) of BRS over a 3 h time course.

All percentages of BRS tested (both AbD Serotec and Pel-Freez BRSs) did not kill *S.* Typhimurium D23580 and LT2 (Fig. 4). The viable bacterial count of *S.* Typhimurium D23580 increased by approximately 1 $\log_{10}$ in all percentages of BRS tested, while *S.* Typhimurium LT2 was bacteriostatic. With *S.* Paratyphi A CVD1901, higher percentages of both AbD Serotec and Pel-Freez BRS (100% and 75%) could kill the bacteria by 1–2 $\log_{10}$ over 180 min (Fig. 4). This antibody-independent killing was removed when BRS was heat-inactivated.

3.3. In the presence of antibody, a high percentage of BRS is required to kill invasive African S. Typhimurium D23580

The difference in susceptibility of the three *Salmonella* isolates to killing by neat and diluted human serum suggested that there will be differences in the amount of BRS required for bactericidal activity in the presence of antibody. Using AbD Serotec BRS as the exogenous complement source and heat-inactivated diluted pooled Malawian serum for antibody, we investigated the amount of BRS required to kill the three bacterial isolates. With *S.* Typhimurium D23580 as the target isolate and 1/40 or 1/400 diluted human sera as antibody source, bacterial growth occurred with 20% BRS, and bacteriostasis with

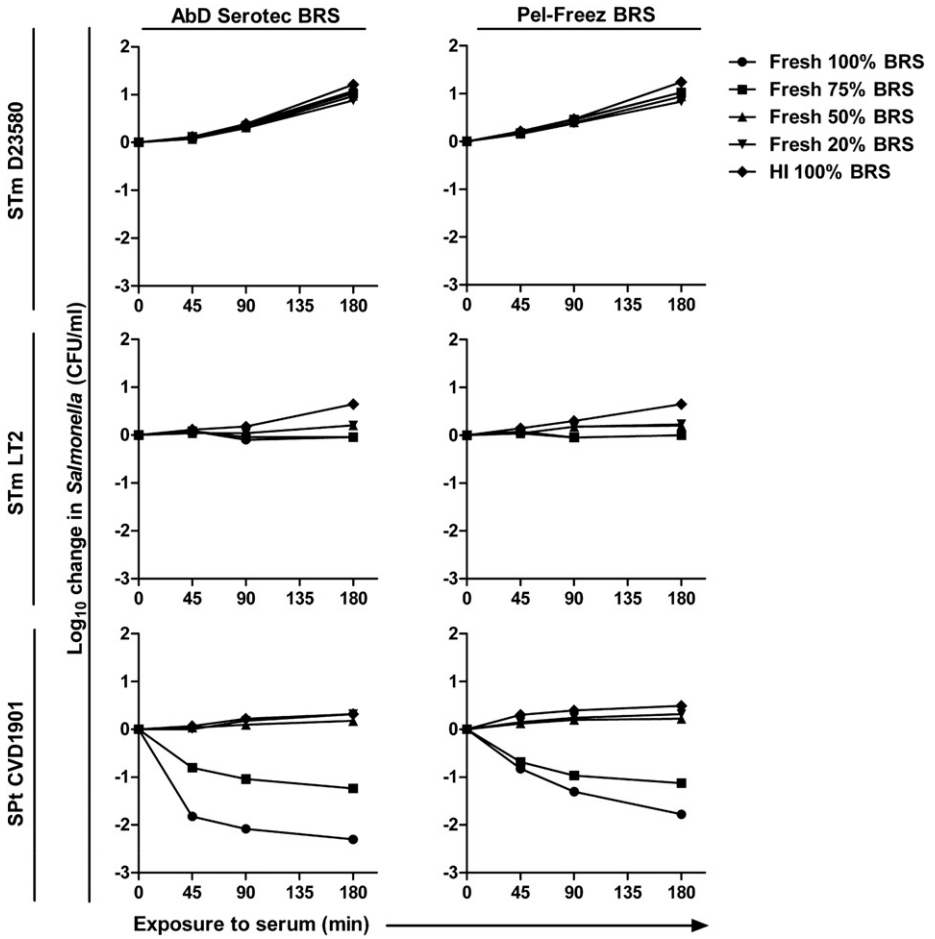

Fig. 4. Bactericidal activity of BRS in the absence of an antibody source. SBA was performed with 1×10^6 *Salmonella*/ml and the number of viable bacteria was determined at t = 45, t = 90, and t = 180 min. *S.* Typhimurium D23580, *S.* Typhimurium LT2 and *S.* Paratyphi A CVD1901 were used as target isolates and killing by different percentages of BRS (AbD Serotec), in the absence of an antibody source, was examined.

50% BRS (Fig. 5). Killing of D23580 occurred with 75% BRS. All three percentages of BRS killed *S.* Typhimurium LT2 at 1/40, 1/400 and 1/4000 diluted human serum, although with limited killing at 1/4000. With *S.* Paratyphi A CVD1901 as the target isolate, all percentages of BRS were bactericidal at all serum dilutions tested (1/4 to 1/40,000) with a 3 $\log_{10}$ kill at lower serum dilutions (1/40 and 1/400) at 180 min and more limited killing at higher serum dilutions. Heat-inactivation of the BRS removed bactericidal activity. For all three isolates, 1/4 diluted human serum gave reduced or no bactericidal activity which appears to be a prozone effect (Lieberman et al., 1988; Zollinger and Mandrell, 1983). Similar results were obtained when the assay was repeated with BRS from Pel-Freez (Fig. A.1). The findings indicate that the amount of BRS used in serum bactericidal assay is critical and that the amount of BRS needed for killing is dependent on the target bacterial isolate.

To verify that the observations made were not specific to the pooled Malawian serum used, we repeated the assay using two sera from 2 healthy individuals (1 European and 1 Asian) as the antibody source (donor 1 and 2). The bactericidal activity of the three sera against the three *Salmonella* isolates was similar across the three BRS percentages tested (Figs. A.2–A.3).

4. Discussion

One method to detect functional antibodies in vaccinated or non-vaccinated human individuals by SBA is to use fresh undiluted human sera as both antibody and complement source. One advantage is that it is the most physiological and closest to 'real-life' scenario of bacteria in the bloodstream during invasive disease. However, sera from vaccinated individuals are often limited in quantity and are not necessarily handled to preserve complement integrity. Whole serum SBA does not permit the determination of a bactericidal titer, the minimum dilution of serum that can kill bacteria. Here, we examined the serum bactericidal activity of diluted fresh human serum against *S.* Typhimurium D23580, *S.* Typhimurium LT2 and *S.* Paratyphi A CVD1901. Our findings indicate that endogenous complement in diluted human sera can be limiting in a SBA against *Salmonella.* A 1/4 dilution of the human sera removed the bactericidal activity against *S.* Typhimurium D23580. This is

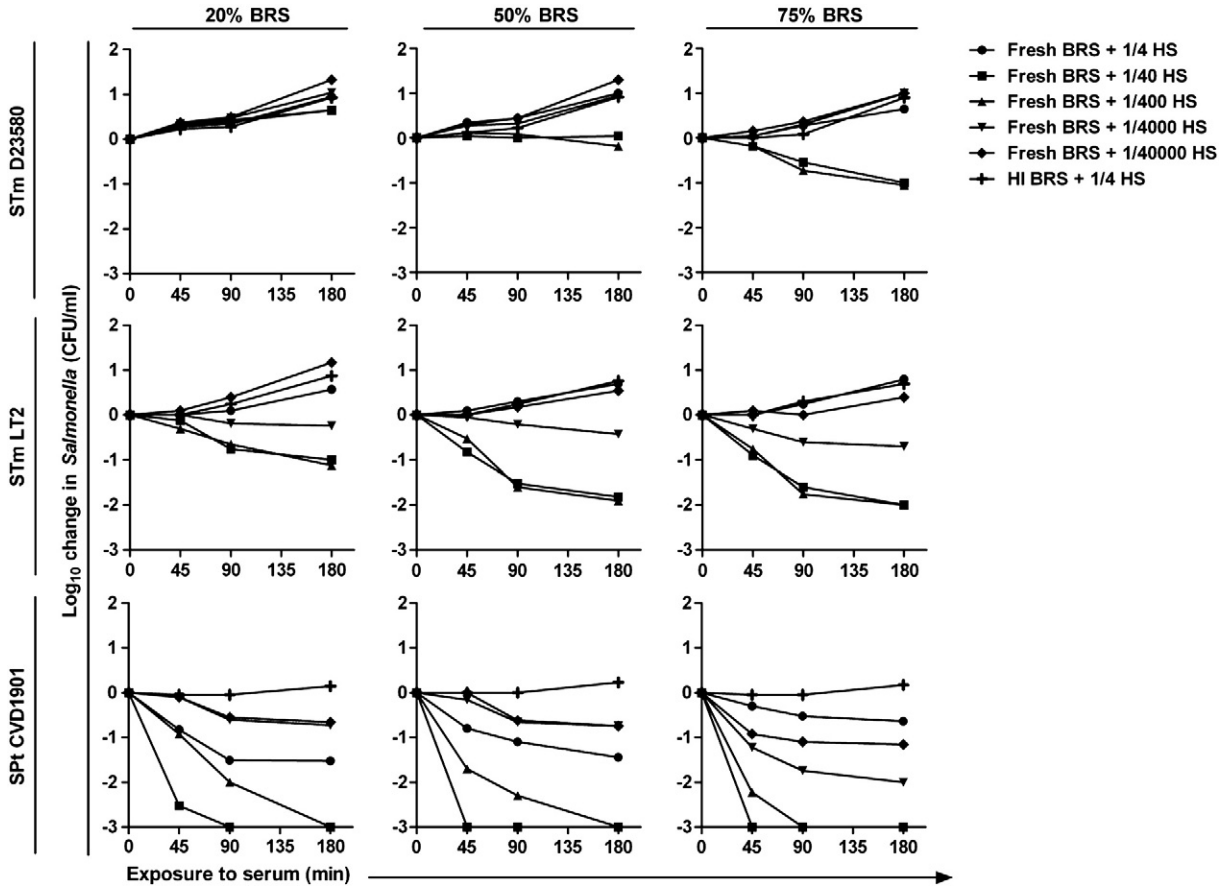

Fig. 5. BRS requirement of *Salmonella* SBA using pooled human serum from Malawian adults as antibody source and AbD Serotec BRS as complement source. SBA were performed with 1×10^6 *Salmonella*/ml and the number of viable bacteria was determined at t = 45, t = 90, and t = 180 min. *S.* Typhimurium D23580, *S.* Typhimurium LT2 and *S.* Paratyphi A CVD1901 were used as target isolates and serum from pooled Malawian adults was used as antibody source. The human serum (HS) was first heat-inactivated (HI) and then diluted (1/4, 1/40, 1/400, 1/4000, 1/40000). BRS from AbD serotec was used as the exogenous source of complement (at 20%, 50% or 75%).

consistent with our previous data where 10% human serum (a 1/10 dilution) was insufficient to effect bactericidal activity against *S.* Typhimurium D23580 (MacLennan et al., 2008). Therefore, an exogenous source of complement is required when diluted human sera are used. Furthermore, if testing the efficacy of antibody to *Salmonella* generated in mice, SBA require an exogenous source of complement. This is because there is an absence of bactericidal activity in mouse sera due to impaired complement function (Siggins et al., 2011).

As most human sera contain naturally-acquired anti-*Salmonella* antibody, it is difficult to obtain human sera lacking anti-*Salmonella* antibody to use as an exogenous source of complement for SBA. Readily available BRS has been commonly used as the source of complement in SBA. BRS does not contain anti-*Salmonella* antibodies, making it an appropriate source of complement for *Salmonella* SBA. We evaluated the optimum BRS requirement for *Salmonella* SBA. Using human sera, which included adults from Malawi where NTS infections are common, we found that the amount of complement used in *Salmonella* SBA is critical and is dependent on the target bacterial isolate. While 20% BRS is sufficient to effect bactericidal activity against *S.* Typhimurium LT2 and *S.* Paratyphi A CVD1901, 75% BRS is needed to effect bactericidal activity against *S.* Typhimurium D23580. *S.* Paratyphi A CVD1901 is the most sensitive of the isolates tested to serum killing. It has been published that Rck, an outer membrane protein encoded on the virulence plasmid of *S.* Typhimurium, binds to complement regulatory protein factor H, thus inhibiting the complement activation *via* the alternative pathway (Ho et al., 2010). Both *S.* Typhimurium D23580 and LT2 have the virulence plasmid harboring the *rck* gene (MacLennan et al., 2008; Rychlik et al., 2006), which might confer the two *S.* Typhimurium isolates protection against complement killing *via* the alternative pathway in the absence of antibody, while still remaining susceptible to complement killing *via* the classical pathway in the presence of antibody. Unlike *S.* Typhimurium, *S.* Paratyphi A lacks the virulence plasmid and hence lacks the *rck* gene (Baumler et al., 1998). The absence of the *rck* gene in *S.* Paratyphi A might result in greater sensitivity to serum killing and would explain why BRS alone in the absence of specific *S.* Paratyphi A antibody could kill the bacteria. Alternatively, since differences in the structure of the O-antigen polysaccharides can affect complement deposition, such differences could account for the variation in susceptibility to killing (Jimenez-Lucho et al., 1987). These

suggest a role for the alternative pathway in *in vitro* serum bactericidal activity against *S.* Paratyphi A, which is insufficient to effect *in vitro* serum bactericidal activity against *S.* Typhimurium (MacLennan et al., 2008).

A potential clinical implication of the finding that a high complement level is needed to effect bactericidal activity against the invasive *S.* Typhimurium D23580 relates to the association of *S.* Typhimurium infections with malaria. This clinical association is well recognized in Africa (Graham et al., 2000; Bronzan et al., 2007). Hypocomplementemia, a marked decrease of serum complement components, is often observed in children and adults with acute malaria (Dulaney, 1947; Siddique and Ahmed, 1995). Hypocomplementemia in African patients with malaria may therefore increase susceptibility to *S.* Typhimurium, giving rise to co-infection with malaria and *Salmonella*.

These findings have clinical implications in the development of a vaccine for *S.* Typhimurium infections in Africa. We demonstrated that the same parameters for SBA cannot be applied to all bacterial isolates. SBA using low amounts of complement would not be sensitive enough to detect bactericidal activity against the invasive African *S.* Typhimurium isolate D23580. This has practical implications for SBA used during preclinical studies that are aimed at gauging potential *in vivo* protection and also for SBA with sera from clinical trials that are aimed at providing information about protection in humans. Hence, this work facilitates the implementation of a flexible SBA that can assess responses to multiple *Salmonella* isolates and aid the development of a vaccine to this deadly pathogen.

Acknowledgments

We are grateful to Myron Levine and the Center for Vaccine Development, University of Maryland, for providing *S.* Paratyphi A CVD1901 and to Robert Heyderman and the Malawi-Liverpool-Wellcome Trust Clinical Research Programme for *S.* Typhimurium D23580. We thank Adam Cunningham for his helpful comments on the manuscript.

Appendix A

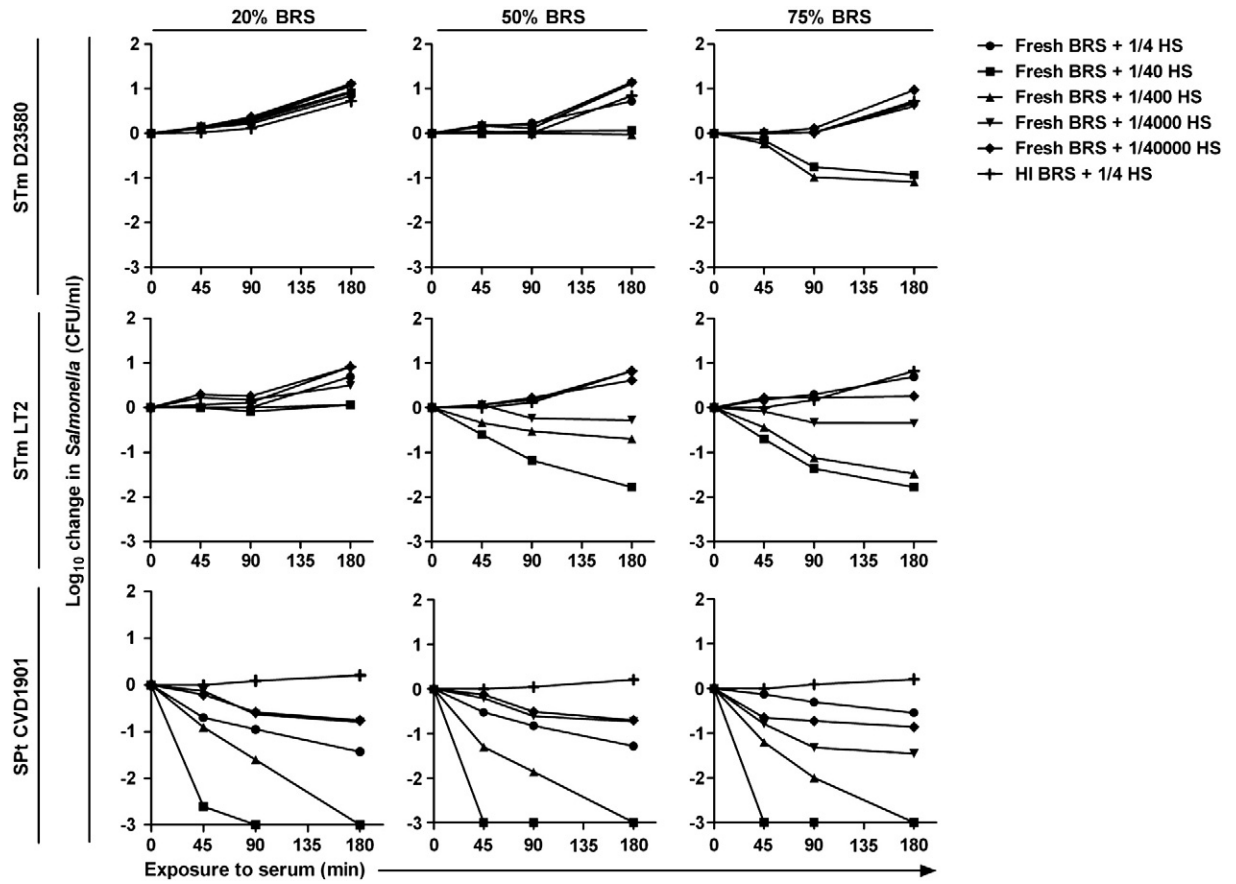

Fig. A.1. BRS requirement of *Salmonella* SBA using pooled human serum from Malawian adults as antibody source and Pel-Freez BRS as complement source. SBA were performed as described in Fig. 5.

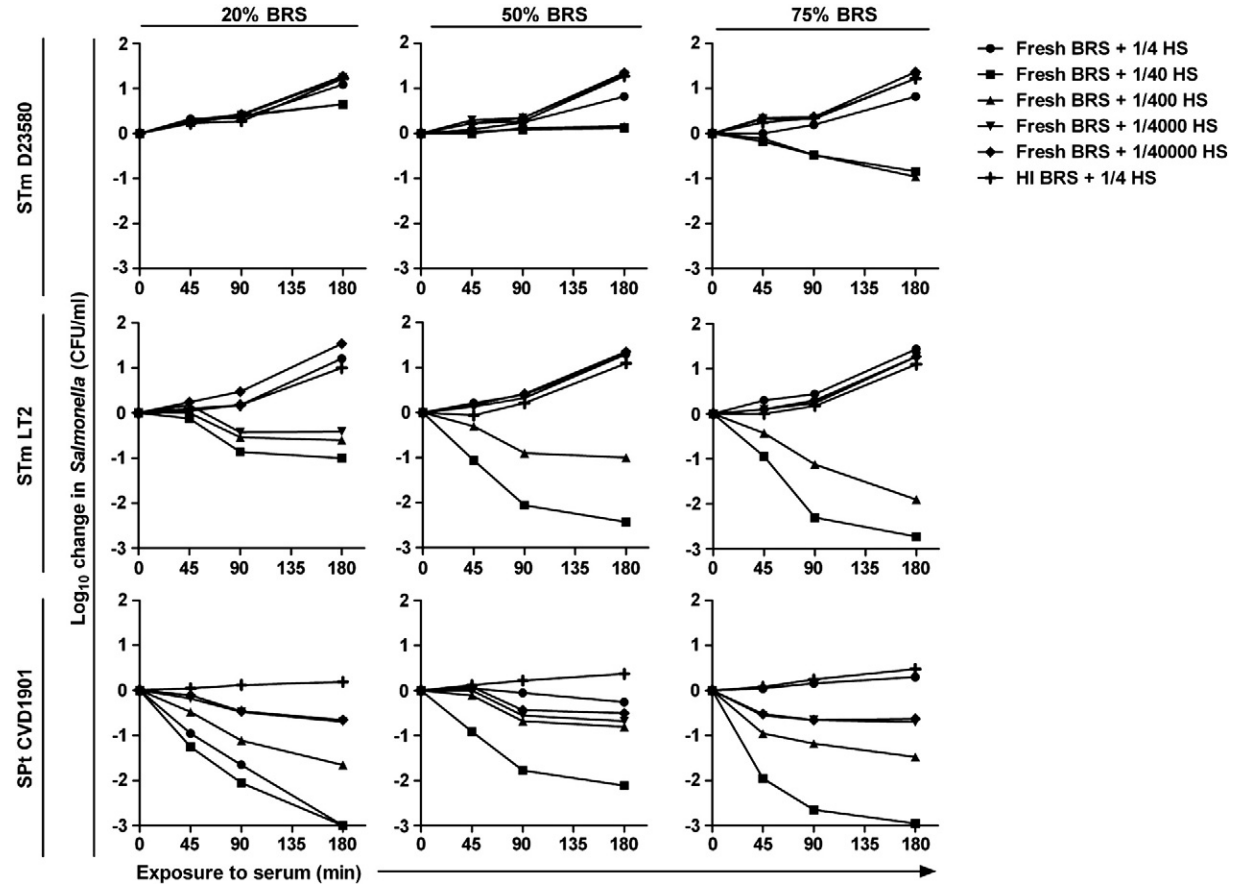

Fig. A.2. BRS requirement of *Salmonella* SBA using serum from donor 1 as antibody source and AbD Serotec BRS as complement source. SBA were performed as described in Fig. 5.

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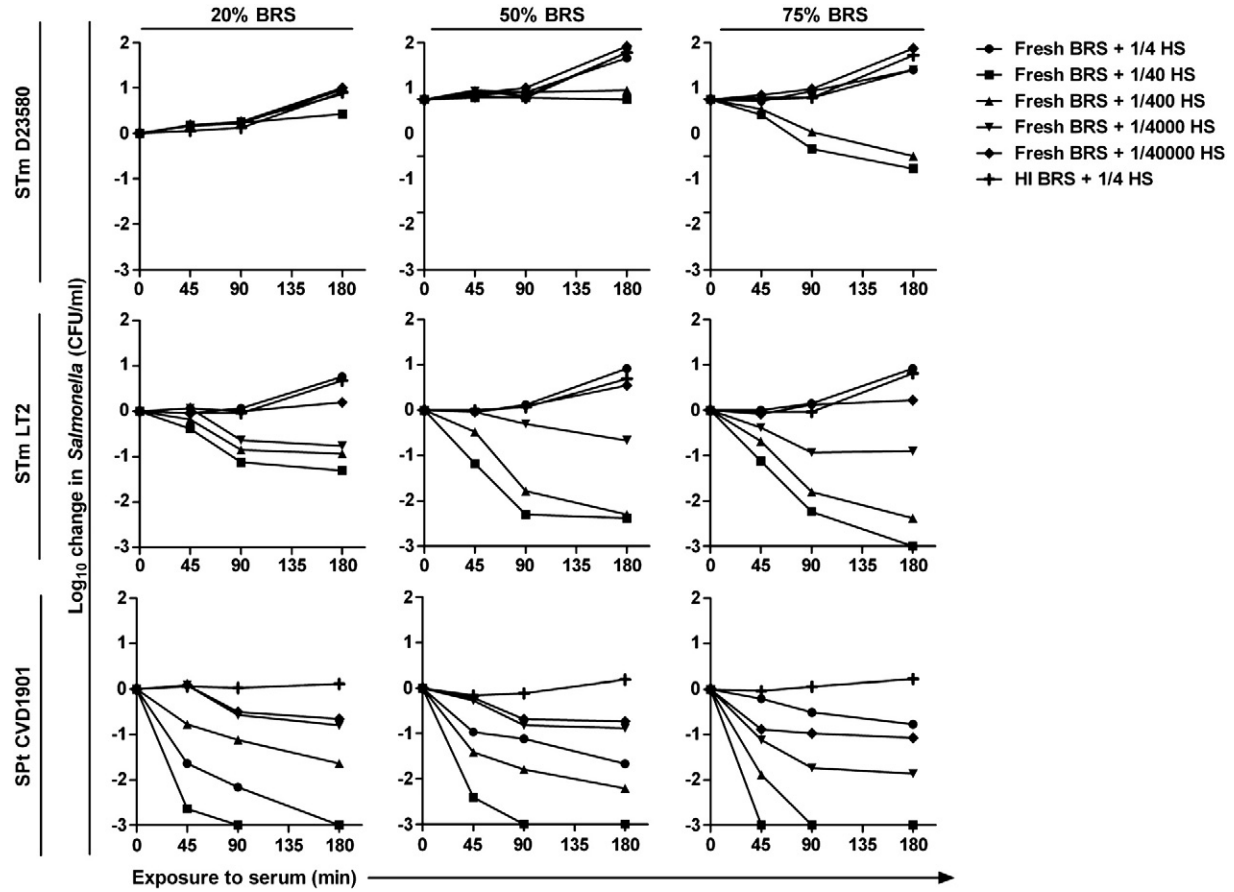

Fig. A.3. BRS requirement of *Salmonella* SBA using serum from donor 2 as antibody source and AbD Serotec BRS as complement source. SBA were performed as described in Fig. 5.

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