Check for updates

METHOD ARTICLE

REVISED **Performance of molecular methods for the detection of**
Salmonella **in human stool specimens** [version 2; peer review:
2 approved]

Angeziwa Chunga Chirambo [1-3], Tonney S. Nyirenda[1,2], Ndaru Jambo[1-3],
Chisomo Msefula [1,2], Arox Kamng'ona [1,4], Sandra Molina [5],
Wilson L. Mandala[6], Robert S. Heyderman [7], Miren Iturizza-Gomara[3],
Marc Y.R. Henrion [1,8], Melita A. Gordon [1,3]

[1]Malawi-Liverpool-Wellcome (MLW) Programme, Blantyre, +265, Malawi
[2]Pathology Department, College of Medicine, Malawi, Blantyre, Malawi
[3]Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Liverpool, L69 3BX, UK
[4]Biomedical Sciences Department, College of Medicine, Malawi, Blantyre, Malawi
[5]London School of Hygiene and Tropical Medicine, London, WC1E 7HT, UK
[6]Malawi University of Science and Technology, Thyolo, +265, Malawi
[7]Research Department of Infection, Division of Infection and Immunity, University College London, London, WC1E 6EJ, UK
[8]Department of Clinical Sciences, Liverpool School of Tropical Medicine, Liverpool, L3 5QA, UK

Abstract

Background: The relationship between asymptomatic *Salmonella*
exposure within the gastrointestinal tract and *Salmonella* bacteraemia
is poorly understood, in part due to the low sensitivity of stool culture
and the lack of validated molecular diagnostic tests for the detection
of *Salmonella* in the stool. The study aimed to determine a reliable
molecular diagnostic test for *Salmonella* in stool specimens.
Methods: We optimised an in-house monoplex real-time polymerase
chain reaction (PCR) for the detection of *Salmonella ttr* and *InvA* genes
in stool by including a selenite broth pre-culture step for *Salmonella*
before DNA extraction and validated their specificity against other
local common pathogens. Then we assessed their performance
against a well-validated multiplex PCR targeting the same *ttr* and *InvA*
genes and against stool culture using clinical stool specimens
collected from a cohort of 50 asymptomatic healthy Malawian children
that were sampled at 1-month intervals over 12 months. We employed
a latent Markov model to estimate the specificities and sensitivities of
PCR methods.
Results: *Ttr* and *InvA* primers were both able to detect all the different
Salmonella serovars tested and had superior limits of detection when
DNA was extracted after selenite pre-culture. T*tr* sensitivity and
specificity for monoplex-PCR were (99.53%, 95.46%) and for multiplex-

Open Peer Review

Reviewer Status ✓ ✓

	Invited Reviewers	
	1	**2**
version 2 (revision) 04 May 2021	✓ report	✓ report
	↑	↑
version 1 09 Oct 2020	? report	✗ report

1. **JY Zhang** , National Institute for Communicable Disease Control and Prevention, Beijing, China

2. **Qiuchun Li** , Yangzhou University, Yangzhou, China

Any reports and responses or comments on the article can be found at the end of the article.

PCR (90.30%, 99.30%) respectively. *InvA* specificity and specificity for using monoplex-PCR was (95.06%, 90.31%) and multiplex-PCRs (89.41%, 98.00%) respectively. Sensitivity and specificity for standard stool culture were 62.88% and 99.99%, respectively. Culture showed the highest PPV (99.73%), and monoplex-*ttr* had the highest NPV (99.67%).
Conclusion: Test methods demonstrated high concordance, although stool culture and monoplexed *ttr* primers had superior specificity and sensitivity, respectively. The use of selenite pre-enrichment step increased *Salmonella* detection rate. Taken together, molecular detection methods used here could be used to reveal the true extent of both asymptomatic and symptomatic *Salmonella* exposure events.

Keywords
Salmonella Typhi, nontyphoidal Salmonella, bacteremia, gastrointestinal tract, diagnostics, stool culture, polymerase chain reaction

This article is included in the Malawi-Liverpool Wellcome Trust Clinical Research Programme gateway.

Corresponding author: Angeziwa Chunga Chirambo (achirambo@mlw.mw)

Author roles: Chirambo AC: Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Visualization, Writing – Original Draft Preparation; **Nyirenda TS**: Conceptualization, Funding Acquisition, Supervision, Validation, Writing – Review & Editing; **Jambo N**: Investigation, Writing – Review & Editing; **Msefula C**: Methodology, Writing – Review & Editing; **Kamng'ona A**: Supervision, Writing – Review & Editing; **Molina S**: Supervision; **Mandala WL**: Conceptualization, Writing – Review & Editing; **Heyderman RS**: Conceptualization, Funding Acquisition, Writing – Review & Editing; **Iturizza-Gomara M**: Supervision, Writing – Review & Editing; **Henrion MYR**: Data Curation, Formal Analysis, Methodology, Software, Writing – Review & Editing; **Gordon MA**: Conceptualization, Funding Acquisition, Resources, Supervision, Validation, Writing – Review & Editing

<div style="border">

REVISED **Amendments from Version 1**

Revisions have been made to this manuscript to address comments raised by the reviewers. We have specifically

- Added a table (Table 4) to summarise the characteristics of the test methods being evaluated here

- Added specific details on *Salmonella* stool culture and Taqman Array Card assay

- Added more background information on the specificity of *ttr* and *InvA*

- Added information on the genomic prevalence of *ttr* and *InvA* gene in different *Salmonella* serotypes

- Provided more information on the statistical analysis used

- Proofread the manuscript and revised accordingly

- Clearly stated the limitations and conclusion

Any further responses from the reviewers can be found at the end of the article

</div>

Introduction

Salmonellae cause a huge global burden of morbidity and mortality. They are globally estimated to be responsible for 300,000 deaths[1–4]. *Salmonella enterica* serovars Typhi and Paratyphi A are the predominant cause of invasive *Salmonella* infections in south and southeast Asia and cause between 129,000 to 223,000 global deaths per year[1,3,5]. In contrast, non-Typhoidal *Salmonella* (NTS) serovars, principally *S. Typhimurium* and *S. Enteritidis*, are a common cause of invasive disease in sub-Saharan Africa (SSA)[4,6]. In 2017, NTS caused an estimated 535,000 cases, with SSA having the highest incidence[7]. Risk factors for invasive NTS (iNTS) disease include young age, recent malaria, and advanced HIV disease. Case fatality rates for iNTS in young children, people infected with HIV, and living in the SSA region were estimated at 13.5%, 41.8%, and 15.8%, respectively[4]. This is in marked contrast to the presentation of *Salmonella* disease in high-income countries, where NTS typically cause a self-limiting diarrhoeal disease in healthy individuals, while bloodstream or focal infections are rare and mainly occur in individuals with specific risk factors such as diabetes, neoplastic and autoimmune disease, or immunosuppressive therapy[8]. However, it is notable in both settings that invasive NTS disease in adults and children is not always associated with diarrhoea[9].

We previously described in under-five-year-old children the sequential development of cellular and humoral immunity against the *Salmonella* serovars causing iNTS disease, and that acquisition of this immunity is associated with decreasing incidence of disease[10,11], suggesting that this immunity is protective. Previous studies have reported that healthy young children experience transient asymptomatic episodes of gastrointestinal infection with non-typhoidal *Salmonella*[12,13], and we, therefore, hypothesise that episodes of asymptomatic *Salmonella* exposure in the healthy gastrointestinal tract during early childhood may facilitate the development of protective immunity. Balanced against this beneficial effect of exposure, diarrhoeal

disease results from enteric *Salmonella* exposure, and invasive NTS disease also follows episodes of asymptomatic gastrointestinal exposure in susceptible children, including those with malaria or malnutrition, or immunocompromised individuals.

Elucidating the relationship between *Salmonella* exposure events within the gastrointestinal tract and resultant Salmonella immunity or Salmonella disease is critical for understanding iNTS disease pathogenesis. Lack of affordable and rapid diagnostic tools for the detection of bloodstream and intestinal *Salmonella* disease hampers our understanding of *Salmonella* disease epidemiology and pathogenesis. Blood culture is considered the gold standard diagnostic test for *Salmonella* bacteremia and is highly specific but has a number of drawbacks; poor turnaround time of between 2 to 7 days, and low sensitivity of about 20% – 30% for samples collected 7 days post-infection[14–16]. Molecular detection of *Salmonella* in blood also has limited apparent sensitivity, and various assays are in development[13,17].

Stool culture is similarly considered the gold standard test for the detection of *Salmonella* in the intestinal tract. However, stool culture, even for diarrhoeal disease when the bacterial load is likely to be high, has poor sensitivity (<50%), and is labour and time-consuming[18]. Real-time PCR has a short turnaround time and is potentially highly sensitive compared to standard culture, and has the capacity for automation and testing for multiple targets[19]. However, stool PCR test performance is hindered by PCR inhibitors and a large number of genetically closely related enteric bacteria. These pose a challenge in generating highly specific and sensitive primers for real-time PCR (qPCR) for *Salmonella*. Furthermore, a lower infective load of *Salmonella* colonisation during asymptomatic infection may further limit detection by PCR.

With this background, we validated an in-house monoplex qPCR method for the detection of *Salmonella* in stool specimens and compared them with a validated multiplex-based qPCR and standard stool culture. Both qPCR assays used primers and probes based on the *Salmonella* tetrathionate respiration gene (*ttr*) and the *Salmonella* invasion gene A (*InvA*). Stool specimens were collected from healthy, mainly asymptomatic healthy Malawian children aged 6–18 months. Assessing a diagnostic test's performance is challenging when the existing "gold standard" test being used has known low sensitivity or specificity. Statistical methods, such as the Latent Markov model, are used to assess diagnostic tests' performance without assigning a gold standard test. Since the current reference standard is known to lack sensitivity, we employed a latent Markov model to estimate the specificities and sensitivities of PCR methods without assigning a gold standard.

Methods

Description of study participants and specimens

Stool specimens collected from a longitudinal cohort of children aged 6 – 18 months recruited from Zingwangwa Health Centre (ZHC) in Blantyre, Malawi, were used to compare the performance of molecular and standard culture for detection of *Salmonella* in stool. The primary study started recruitment

in August 2013, and follow-up was concluded in December 2014[17]. Group sensitisation of the study by well-trained study nurses was done to parents or guardians of six-month-old children attending a vaccination clinic at ZHC. Individual sensitisation was also done to parents or guardian that were interested in joining the study. Children who met the inclusion criteria of being healthy were recruited into the study after obtaining consent. Children born preterm (less than 38 weeks gestation), HIV positive or HIV exposed, and those with fever >38°C or any acute illness were excluded from the study[20].

Stool samples were collected monthly until they were aged 18 months. Stool specimens were collected in sterile and clean containers and transported to the laboratory on the same day. From 60 children recruited at 6 months of age, 10 children withdrew from the study, and 600 stool specimens were collected and tested by culture on the day of sample collection at the College of Medicine and Malawi Liverpool Wellcome Laboratory. Molecular tests were done on frozen samples that were available at the time the tests were done.

Salmonella stool culture
A matchstick head-size sample of stool was inoculated in 10 ml of selenite F broth (Oxoid, UK, catalog number: 2300631) and aerobically incubated overnight at 37 °C for 18–24 hours. The top layer (1 ml) of an overnight culture was spun at 20,000 g for 5 minutes. This is a method that has been developed in our laboratory. A 1 ul loop was used to subculture *Salmonella* from the pellet by spreading on Xylose Lysine Deoxycholate (XLD) agar (Oxoid, UK, catalog number: 2547703) to achieve single colonies. Careful plate spreading prevents overcrowding of colonies. A single colony was picked and cultured on sheep blood agar. An aliquot of the selenite broth was also frozen for molecular detection. A single colony of presumptive *Salmonella* was cultured onto sheep blood agar (Oxoid, UK, catalog number: 2910831) and MacConkey agar plates (Oxoid, UK, catalog number: 2529552) and incubated aerobically at 37°C for 18–24 hours. *Salmonella colonies* were then distinguished from other enteric bacteria (i.e., *Citrobacter* and *Serratia*) using triple sugar iron agar (Oxoid, UK, catalog number: 1882283) and Urea agar (Oxoid, UK, catalog number: 1779617) biochemical tests. Further *Salmonella* identification was determined using API® 10S (bioMérieux, France, catalog number: 1007181060) according to the manufacturer's instructions.

Monoplex-qPCR *ttr* and *InvA* assay
Validation of the monoplex- qPCR ttr and InvA assay. For the monoplex-qPCR, the *ttr* primers and probe were designed and validated by Federal Institute for Risk Assessment, Berlin, Germany, according to the published DNA sequence of the *S. enterica* serotype Typhimurium *ttr* locus for *Salmonella* detection (GenBank accession no. AF282268). The use of *ttr* was based on 21. In this study, the ttr gene's specificity was assessed using 110 *Salmonella* strains representing 31 serotypes, and it demonstrated 100% specificity. The *InvA* gene has been widely studied and used as a pan *Salmonella* marker. In 22, the specificity of the *InvA* gene was assessed using 242 *Salmonella*

strains representing 43 serotypes. It also demonstrated 100% specificity. *InvA* has shown 100% specificity in several other studies. Both primers required optimisation for use in stool specimens. The DNA sequences of all the primers and probes used in this study are listed in Table 1.

Specificity of *ttr* and *InvA* primer/probe set for *Salmonella* compared to other local pathogens
To determine the prevalence of ttr and *InvA* in the genomes of *Salmonella* serotypes, NCBI nucleotide blast was conducted (10 February 2021). Ttr and *InvA* nucleotide sequences were used as the query sequence against *Salmonella* enterica subspecies enterica (taxid:59201) genomes with a maximum target sequence set at 1000. Both primer sequences demonstrated 100% identity for most commonly isolated *Salmonella* serotypes (supplementary material). To determine the specificity of the primers *in vitro*, 9 different locally isolated and whole genome sequenced *Salmonella* strains and 26 pure isolates of non- *Salmonella* bacterial strains locally isolated from blood culture were tested using *ttr* and *InvA* primer/probe sets (Table 2). These 9 *Salmonella* strains represented all known *Salmonella* serotypes in the MLW isolate archive. The non-*Salmonella* strains were chosen because they are genetically closely related to *Salmonella* or because their growing conditions are similar to *Salmonella*. These strains were collected from MLW bacterial blood culture repository. Overnight cultures of the frozen samples were made on SBA or LB agar. One colony was then cultured in liquid media. After reaching stationary growth phase, a known and matched concentration of about 10^6 CFU was used for DNA extraction using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Netherlands, catalog number: 51604) but without the bead beating step. Miles and Misra technique was used for bacteria quantification.

Limits of detection in different conditions A well-characterised invasive *S.* Typhimurium ST313 strain (D23580), isolated from an HIV-negative child in Malawi, and representative of our commonest invasive bloodstream infections, was used as a reference strain for determining limits of detection in varying kinds of sample[23,24]. Three types of Salmonella samples were prepared for comparison using RT-PCR; 1) pure *Salmonella* isolates picked from a blood agar plate, 2) *Salmonella* cultured in selenite broth, and 3) *Salmonella* spiked into stool. *Salmonella* stool spiking was done to determine the inhibitory effect that stool may have on the assay, affecting the limit of detection. For this, a stool sample was collected from a healthy individual and confirmed as *Salmonella* negative by culture. The stool sample was thereafter diluted with PBS (50% w/v) and then spiked with *S.* Typhimurium, D23580, at varying doses of viable bacteria. The viable dose of *Salmonella* was adjusted across a range from 10^0–10^6 CFU/ml and quantified using Miles and Misra technique. DNA was extracted for RT-PCR, as above. All experiments were repeated three times on different days by the same operator.

Detection of Salmonella in clinical samples using monoplex-qPCR ttr and InvA assay. The primer/probe sets were then used to detect *Salmonella* in clinical stool samples collected from the

Table 1. List of primers and probes sequences used in this study. Primers and probes sequences used in this study include in-house designed *InvA, ttr* previously validated for *Salmonella* detection in food, and TAC-*InvA* and TAC-*ttr* used on a well-validated TAC assay as pan *Salmonella* primers.

	Primer name	Primer direction	Primer code/Probe description
1	*INVA*	Forward	5'-AGCGTACTGGAAAGGGAAAG-3'
2	*INVA*	Reverse	5'-CACCGAAATACCGCCAATAAAG-3'
3	*INVA*	Probe	FAM-TTACGGTTCCTTTGACGGTGCGAT-BHQ1
4	*ttr*	Forward	5'-CTCACCAGGAGATTACAACATGG-3'
5	*ttr*	Reverse	5'-AGCTCAGACCAAAAGTGACCATC-3'
6	*ttr*	Probe	FAM-CACCGACGGCGAGACCGACTTT-BHQ1
7	*InvA*-TAC	Forward	5'-GGCAATTCGTTATTGGCGATA-3'
8	*InvA*-TAC	Reverse	5'-CACGGTGACAATAGAGAAGACAACA-3'
9	*InvA*-TAC	Probe	FAM-CCTGGCGGTGGGTT-MGB
10	*ttr*-TAC	Forward	5'-CTCACCAGGAGATTACAACATGG-3'
11	*ttr*-TAC	Reverse	5'-AGCTCAGACCAAAAGTGACCATC-3'
12	*ttr*-TAC	Probe	FAM-CACCGACGGCGAGACCGACTTT-MGB

longitudinal cohort study of healthy asymptomatic children. For the monoplex qPCR, approximately 200µl top layer of frozen Selenite F broth overnight stool culture, or 200 mg of stool, was suspended in 500 µl of PBS. DNA was extracted using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Netherlands, catalog number: 51604) according to the manufacturer's instructions, with an added bead-beating step. Eluted DNA was stored at –20°C.

A previously-optimised in-house PCR protocol was used[17]. Briefly, the master mix for RT-PCR was prepared using pre-defined quantities. A total of 20µl master-mix for each sample was comprised of the following: 12.5µl Platinum ® Quantitative PCR Super Mix-UDG (Life Technologies, USA, Catalog number: 11730025), 0.10µl specific forward primer, 0.10 specific reverse primer, 0.10 specific probe (all primers and probes at 200nM), 0.05µl ROX reference dye (Life Technologies, USA, Catalog number: 12223012) at 50nM final concentration, and 7.15µl nuclease-free water. This mixture was transferred to 96-well plate PCR wells. 5µl of test DNA, positive controls DNA (DNA from D23580), technical extraction negative control, and assay negative control (UV treated water) were added in triplicates to appropriate wells containing 20ul of master-mix. The qPCR was run for 40 cycles using Applied Biosystems® 7500 Real-Time PCR Systems (Life Technologies, USA). The following cycling conditions were used; initial denaturation at 95°C for 1 minute, denaturation at 95°C for 15 seconds, annealing/extension at 60°C for 30 seconds, final extension: 12°C. The threshold was set in the lag phase. An assay was considered to have passed when the positive controls were positive, and both the technical extraction negative and assay

negative controls were negative. Test sample cycle threshold (Ct) values were evaluated after subtracting the baseline value. Samples with cycle threshold (Ct) values of less than or equal to 35 were considered positive.

Detection of *Salmonella* using multiplex qPCR assay

As a comparator, we used a well-validated TAC assay on DNA extracted from stool samples, according to the manufacturer's protocol. The customised Taqman Array Card assay developed and validated at the University of Virginia was used. The performance of the TAC method has been previously described and has now been widely used[12,25–27]. It is used to detect multiple enteric pathogens, including bacteria, viruses, protozoa, and helminths. Targets included on the TAC card for pan *Salmonella* detection are *InvA* and *ttr*[28]. Phocine Herpesvirus (PHhv) and MS2 targets are included as internal positive controls.

To extract total nucleic acid (TNA) from the clinical samples for TAC assay, we used QIAamp Fast DNA Stool Mini Kit (QIAGEN, Netherlands, catalog number: 51604) - the same DNA extraction kit and protocol that were used to extract whole-stool DNA for the monoplex qPCR assay, with the addition of internal extraction positive controls. For TNA extraction, each sample was extracted together with internal positive controls, Phocine Herpesvirus (PHhv), and MS2. PHhv and MS2 were added to the inhibitX buffer before being added to each sample, as previously described[28]. An assay was considered to have passed when both MS2 and PHhv internal positive (amplification crossing the threshold) and negative controls (no amplification crossing the threshold line) passed and when the sample had a sigmoid curve that crossed the threshold line. A sample was

Table 2. Bacterial organisms tested for the specificity of *ttr* and *InvA* primer/ probe sets. Bacterial organisms used in this study to test for the specificity of *ttr* and *InvA* primer/ probe sets. Nine *Salmonella* and 26 non-*Salmonella* isolates previously isolated at MLW laboratory were retrieved and tested either as direct or selenite sub-cultured isolates.

Bacteria isolates	Number tested	Direct		Selenite sub-cultured	
		ttr Positive	*InvA* Positive	*ttr* Positive	*InvA* Positive
Morganella morgana	1	0	0	0	0
Streptococcus pneumonia	1	0	0	0	0
Staphylococcus aureus	1	0	0	0	0
Citrobacter	1	0	0	0	0
Klebsiella	1	0	0	0	0
Enterobacter	1	0	0	0	0
Acinetobacter	1	0	0	0	0
Enterobacter intermedius	1	0	0	0	0
Enterococcus feacium	1	0	0	0	0
E. coli	17	0	0	0	0
S. Typhi	1	1	1	1	1
S. Typhimurium	1	1	1	1	1
S. Enteritidis	1	1	1	1	1
S. Braenderup	1	1	1	1	1
S. Virchow	1	1	1	1	1
S. Bonn/Fann	1	1	1	1	1
S. Oesterbro/Zanzibar	1	1	1	1	1
S. Heidelberg	1	1	1	1	1
S. Dublin	1	1	1	1	1

classified as pathogen positive at a Ct value of <35. Only results for *Salmonella* are reported here.

Statistical analysis

Data were recorded and analysed in MS Excel (version 16.14.1 (18061302)). Sensitivities and specificities of the different PCR methods were estimated using a latent Markov model (LMM)[29]. We have previously described the LMM and various extensions that we considered for modeling longitudinal diagnostic test data[30]. We implemented the LMM within a Bayesian framework using R (version v3.5.1) and JAGS (version 4.3.0) via the rjags (version 4.6) R package[31]. LMMs have been extensively used for discrete-time longitudinal data in the absence of a gold standard diagnostic procedure[32,33]. LMMs consists of a process model for a latent condition (in our case, the unobserved true infection status) evolving over

time and a measurement model for the observed outputs (in our case, the results from the 5 diagnostic test methods) conditional on the latent state. We considered several LMMs, with and without mixed effects and with either time-homogeneous or time-heterogeneous transition matrices[30]. Convergence and identifiability of the LMM were checked by inspecting trace plots and computing Gelman-Rubin potential scale reduction factors[34]. The more complex models exhibited poor mixing or convergence of MCMC chains (most likely due to the sparse number of positive samples). As a result, the LMM we used for this dataset is a basic LMM with no random effects and a time-homogeneous transition matrix. To report positive predictive values (PPV) and negative predictive values (NPV), we calculated an estimate of the infection prevalence. For the Bayesian LMM, we report maximum *a posteriori* (MAP) parameter estimates together with 95% credible intervals (CrI),

specifically the highest posterior density intervals (HDI) with 95% coverage. All other analyses report (frequentist) parameter estimates and corresponding 95% confidence estimates (CI).

Ethical considerations

Ethical approval for this work was granted by the University of Malawi, College of Medicine Research Ethics Committee (P.01/13/1327). Written informed consent was obtained from the parent or guardian of each participating child.

Results

Ttr and *InvA* primers for Salmonella do not cross-react with closely related enteric micro-organisms

We first validated the *ttr* and *InvA* primers that were used in the monoplex-qPCR assay by assessing the sensitivity and specificity of the primers for Salmonella, using a standardised number of 10^0–10^6 CFU/ml of 9 different locally-relevant *Salmonella* strains and 26 non- *Salmonella* bacterial strains as indicated in Table 2. We included 17 strains of *E. coli* because of the close genomic relatedness of *Salmonella* and *E. coli*. Bacterial isolates enriched in Selenite F broth (referred here as selenite sub-cultured) or not (referred here as direct culture) were used in this evaluation. We found that *ttr* and *InvA* assays both achieved 100% sensitivity and specificity either as direct isolates or selenite sub-cultured isolates. Table 2 demonstrates that all *Salmonella* strains tested positive with both monoplexed primer pairs, and all other bacterial strains were negative, confirming a lack of cross-reactivity. Additionally, the NCBI nucleotide blast demonstrated that the genomic prevalence of *ttr* and *InvA* genes is high. Both primer sequences demonstrated 100% identity for most commonly isolated Salmonella serotypes.

Selenite broth culture enhances the detection of Salmonella in stool using either *ttr* or *InvA* primers

The limits of detection (LOD) of qPCR for *Salmonella* were then determined using *S.* Typhimurium strain D23580 serially diluted and tested as direct isolates, selenite broth cultured samples, or isolates spiked into a culture-negative stool specimen. We found that limits of detection for *ttr* were 1, 10, and 100 CFU/ml, and for *InvA* were 1, 100, and 100 CFU/ml for selenite sub-cultured broth, direct isolates, and stool-spiked isolates, respectively, with 98.5% qPCR efficiency for *ttr* and 97.2% qPCR efficiency for *InvA*. No statistically significant difference was observed in the LOD when *ttr* was compared with *InvA* in either direct isolates (p = 0.3212), selenite sub-cultured samples (P = 0.2534), or *salmonella* spiked stool samples (P = 0.2361). Importantly, we found that the *ttr* assay was significantly different when direct isolates (LOD = 10 CFU/ml) were compared with selenite sub-cultured samples (LOD = 1 CFU/ml) (p<0.0001), and when selenite sub-cultured isolates were compared to *Salmonella* spiked stool (*p* <0.0001), and there was no significant difference when direct isolates were compared to *Salmonella* spiked stool (*p*=0.2965).

Similarly, we found that detection in *InvA* qPCR assay direct isolates was significantly different compared to selenite broth cultures isolates (*p* < 0.0001), and selenite subculture isolates were also significantly different from *Salmonella* spiked stool (*p* < 0.0001). In contrast, there was no significant difference

between direct isolates compared to *Salmonella* spiked stool samples (*p* = 0.2862). In summary, we found that selenite broth overnight liquid culture of stool samples enhanced the molecular detection of *Salmonella* using either *ttr* or *InvA* primers, even if culture of the broth remained negative.

Ttr and *InvA* primers had both high specificity and sensitivity rates, while stool culture had high specificity but low sensitivity

The samples from healthy children were used to determine the performance of stool culture, monoplex *ttr*, monoplex *InvA*, multiplex TAC *ttr*, and multiplex TAC *InvA*. Standard stool culture was performed on a total of 600 specimens at different time points. Molecular tests were used to detect *Salmonella* in the available 421 stool DNA specimens. We detected *Salmonella* in 23, 40, 29, 56, and 47 of 421 stool specimens, using standard stool culture, *ttr*, *InvA*, TAC-*ttr*, and TAC-*InvA* respectively. Of the 23 *Salmonella* stool culture-positive samples, 21 samples were also positive with either one or more molecular tests, while 2 were molecular tests negative.

Based on a time-homogeneous LMM without random effects (Table 3 and Figure 1A), we report the specificities and sensitivities of the detection methods with their 95% credible intervals (Bayesian confidence intervals). The observed specificity rates from highest to lowest were for stool culture (99.99%), TAC-*ttr* (99.30%), TAC-*InvA* (98.00%), monoplex *ttr* (95.46%), and monoplex *InvA* (90.31%), respectively. The observed sensitivity rates from highest to lowest were monoplex *ttr* (99.53%), monoplex *InvA* (95.06%), TAC-*ttr* (90.30%), TAC-*InvA* (89.41,) and stool culture (62.88%) respectively (Table 3 and Figure 1A). While stool culture achieved the highest specificity and monoplex *ttr* the highest sensitivity, monoplex *ttr* achieved arguably the best sensitivity-specificity trade-off: very high sensitivity (99.53%) at a relatively small drop in specificity (95.46) compared to stool culture.

High negative and positive concordance for stool culture, monoplex *ttr*, monoplex *InvA*, Multiplex *ttr*, and multiplex *InvA*

Next, we explored correlations between stool culture, monoplex *ttr*, monoplex *InvA*, Multiplex *ttr*, and multiplex *InvA*. In this exploration, we considered all test results, whether positive or negative. To account for both censored observations and the longitudinal nature of the data, we calculated repeated measures of correlation coefficients[35] using the ranks of observations for each test (akin to a repeated-measures Spearman correlation coefficient) for measuring the correlation between the Ct values for the four molecular tests and point bi-serial correlation coefficients based on ranks for measuring correlations between standard stool culture and each of the qPCR tests (Figure 2A). The correlation coefficients vary quite widely from 0.12 (monoplex *InvA* and TAC-*InvA*) to 0.8 (stool culture and TAC-*ttr*). Given that for truly negative samples, the Ct values are effectively randomly distributed near the threshold used to discriminate between positive and negative samples and that most samples were negative in most tests, the somewhat weak correlations we observe can be driven by the random Ct values for negative samples. For this reason, using only the binary negative / positive outcomes for each test, we computed

Table 3. Probability estimates of the specificities and the sensitivities, PPV, and NPV of the diagnostic tests.
Maximum a posterior probability estimates of the specificities and the sensitivities, PPV, and NPV of the diagnostic tests. Also reported are the 95% highest density credible intervals for each parameter.

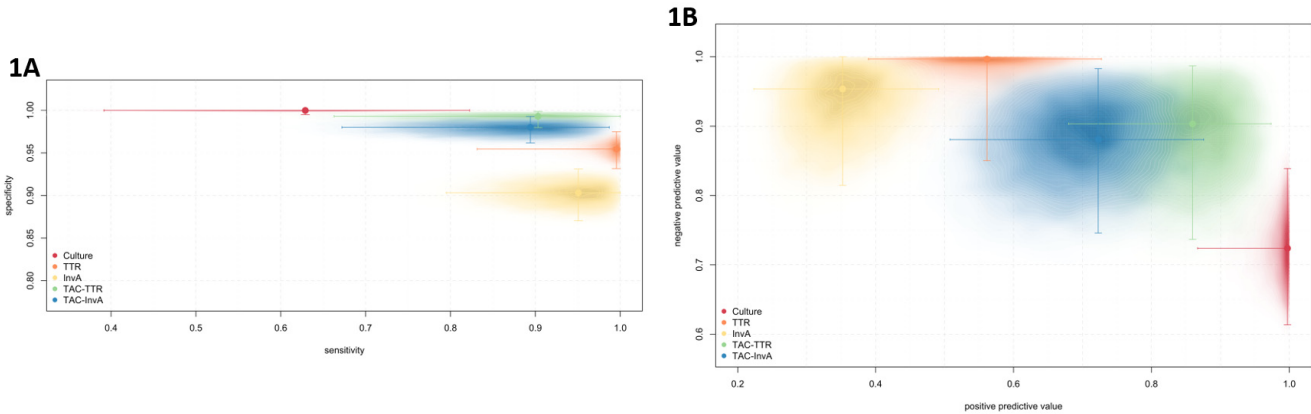|	Sensitivity		Specificity		Positive predictive value		Negative predictive value	
	MAP	(95% CrI)	MAP	95% CrI	MAP	95% CrI	MAP	95% CrI
Stool culture	0.6288	(0.3916,0.8223)	0.9999	(0.9949,10000)	0.9973	(0.8668,10000)	0.7238	(0.6135,0.8389)
ttr	0.9953	(0.8315,1.0000)	0.9546	(0.9317,0.9749)	0.5615	(0.3897,07275)	0.9967	(0.8501,10000)
InvA	0.9506	(0.7950,10000)	0.9031	(0.8702,0.9311)	0.3521	(0.2233,0.4915)	0.9536	(0.8147,10000)
TAC-*ttr*	0.903	(0.6628,10000)	0.993	(0.9797,0.9987)	0.8597	(0.6798,0.9736)	0.9033	(0.7367,0.9869)
TAC-*InvA*	0.8941	(0.6721,0.9869)	0.98	(0.9618,0.9928)	0.7228	(0.5079,0.8757)	0.8807	(0.7459,0.9828)

Figure 1. Maximum a posteriori probability estimates of the specificities and sensitivities (Figure **1A**), positive and negative predictive values (Figure **1B**) together with 95% highest density credible intervals (segments) and posterior density estimates (contours) for stool culture, *ttr*, *InvA*, TAC-*ttr*, and TAC. Big dots and error bars represent the median values and 25 and 75 percentile.
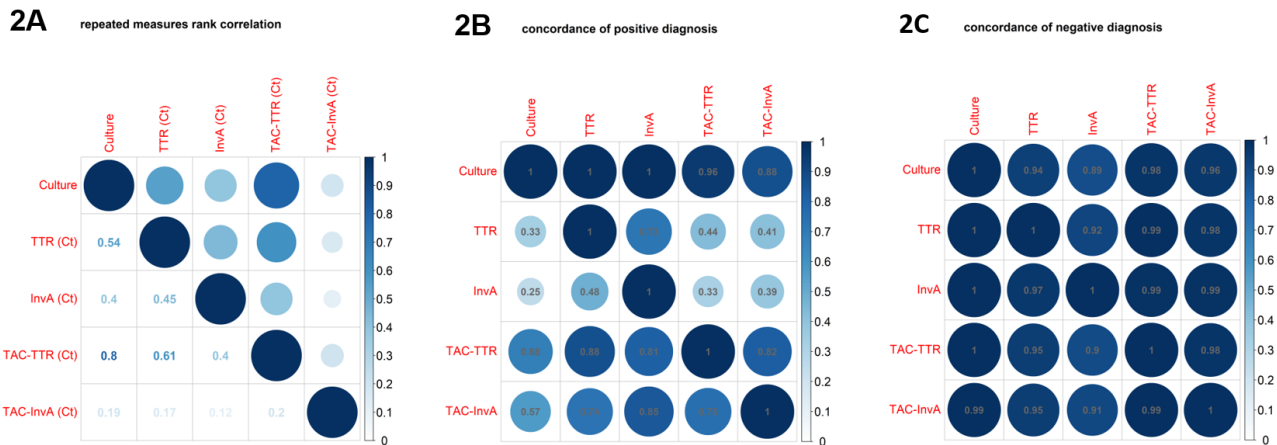
Figure 2. Correlation coefficients for the four molecular tests (using Ct values) and stool culture using positive or negative (Figure **2A**). Concordance coefficients for positive (Figure **2B**) and negative (Figure **2C**) diagnosis obtained using binary negative or positive outcomes for each test. For example, in Figure **2B**, the intersection of the row labeled 'Culture' and the column labeled '*ttr*' lists the proportion of positive test results for the Culture test that are also positive for the *ttr* test. Both the size and colour depth represent the magnitude of correlation.

positive (Figure 2B) and negative (Figure 2C) concordance: for example, in Figure 2B, the intersection of the row labeled '*ttr,*' and the column labeled '*InvA*' lists the proportion of positive test results for the *ttr* test that are also positive for the *InvA* test. Unexpectedly (given that most samples were negative), negative concordance (Figure 2C) was very high, with the lowest negative concordance being 89%. Results for positive concordance (Figure 2B) are also relatively high, though there is more variation, ranging from 25% (for positive *InvA* results confirmed by positive stool cultures) to 100% (positive stool cultures confirmed by positive monoplex *ttr* or positive monoplex *InvA*).

Stool culture had high positive predictive value while molecular tests methods had high negative predictive values

To report PPV and NPV, for an estimate of prevalence, we use the model-estimated stationary (time-homogeneous model) probability of being infected (MAP 5.25%, 95% credible interval [3.27%, 8.14%]). From highest to lowest, the estimated PPVs were culture (99.73%), TAC-*ttr* (85.97%), TAC-*InvA* (72.28%), mono-*ttr* (56.15%), mono-*InvA* (35.21%). From highest to lowest, the estimated NPVs were mono-*ttr* (99.67%), mono-*InvA* (95.36%), TAC-*ttr* (90.33%), TAC-*InvA* (88.07%), and culture (72.38%) as indicated in Table 3 and Figure 1B. While stool culture has the highest PPV (99.73%) and mono-*ttr* the largest NPV (99.67%), a good trade-off between PPV and NPV is achieved by TAC-*ttr* (both high PPV, 85.97%, and high NPV, 90.33%). We note that these PPV and NPV estimates are for an asymptomatic population of children in urban Blantyre, Malawi, and are not directly generalisable to different contexts and populations where prevalence may be higher or lower.

Discussion

The burden of asymptomatic gastrointestinal exposure to *Salmonella,* which could be linked to either the development of immunity or, conversely, to bloodstream infection, is not known due to lack of robust *Salmonella* detection methods for stool specimens. This study aimed to optimise detection methods and to validate and compare the performance of monoplex *ttr* and *InvA* qPCR assays (*ttr* and *InvA*) against *ttr* and *InvA* qPCR assays on a validated multiplex qPCR platform (TAC-*ttr* and TAC-*InvA*), and compare all molecular methods to standard *Salmonella* stool culture. Validation of the monoplex *ttr* and *InvA* primers showed that the primers do not cross-react with other enteric pathogens, and LOD testing showed that selenite pre-culture promotes molecular detection, even when culture is negative. Stool culture demonstrated the highest specificity but low sensitivity than all the molecular tests. Stool culture, despite having low sensitivity, still remains important in *Salmonella* diagnosis. Culture allows for antimicrobial susceptibility testing and strain typing. *Ttr* detected on the monoplex platform demonstrated superior sensitivity to stool culture, *InvA*, TAC-*ttr*, and TAC-*InvA*. All the test methods, however, displayed high concordance to each other.

Several studies have developed *Salmonella* detection methods based on antigen detection or nucleic acid amplification[16,18,36,37]. Both monoplex and multiplex nucleic acid amplification-based detection methods have been developed[38–41]. Most of these have, however, focused on *Salmonella* detection in blood as opposed to stool specimens. Some multiplex qPCRs to specifically detect *Salmonella* and its serovars or for the detection of multiple enteric pathogens in stool specimen (including *Salmonella*) have recently been developed[28,42–44]. The advantage of multiplex qPCR is that it is fast in determining the primary etiological agent in cases where multiple pathogens or different serovars cause the outcome, but it is expensive if one is interested in detecting only one particular pathogen (Table 4). By contrast, the advantage of a monoplex test is that it is economical than the multiplex, faster than stool culture, even with the addition of the selenite enrichment step and allows for batch-processing of samples which increases the efficiency of the test method. In this study, the same primer/ probe sets were tested using both the monoplex and multiplex qPCR platforms. The monoplex qPCR maximised sensitivity, while the multiplex panel provided a balanced pay-off between sensitivity and specificity (Table 4). The high sensitivities of the monoplex qPCR could be attributed to the use of selenite pre-cultured stool as opposed to extraction of DNA from neat stool samples, which is used in the multiplex qPCR. Selenite sub-cultured stool samples were not used on the multiplex platform because the manufacturer's protocol was followed. Other studies have, however, also demonstrated superior performance of monoplex qPCR when compared with multiplex qPCR. The monoplex qPCR is therefore ideal for studies that are only interested in determining the presence or absence of *Salmonella* while capitalising on the sensitivity of the test, while multiplex qPCR will have an added advantage if a study wants to detect multiple pathogens while having a pay-off between sensitivity and specificity.

The *ttr* primer/ probe set used in the monoplex qPCR was previously validated for use in food samples and required validation in stool specimens. Our in-house developed *InvA* primer/ probe set also needed validation. Both assays demonstrated that they could detect all the different *Salmonella* strains, including *S.* Enteritidis, *S.* Typhimurium, and *S.* Typhi strains which are the commonly isolated strains in Malawi and SSA[45]. Comparing the limits of detection of different *Salmonella* isolate conditions demonstrated that selenite pre-culture achieves a significantly lower limit of detection (1 CFU/ml) as opposed to direct isolates (10 CFU/ml) and *Salmonella*-spiked stool (10 CFU/ml). Selenite F broth is a selective broth that suppresses fecal coliforms and streptococci growth to optimise *Salmonella* growth[46]. The LOD achieved after sub-culturing samples in Selenite enrichment broth agrees with results demonstrated by other studies, including a study done by Boer *et al.,* who showed that sub-culturing samples in Selenite F broth promotes the recovery of *Salmonella* in stool samples and improves sensitivity if samples are subsequently tested using molecular methods like PCR[46,47].

Given the lack of a true gold standard diagnostic test, we took a model-based approach and used an LMM to estimate the specificities and sensitivities of the 5 *Salmonella* detection methods. Stool culture demonstrated the highest specificity but had the lowest sensitivity. All molecular assays; TAC-*ttr*,

Table 4. Characteristics of *Salmonella* stool culture, Monoplex -qPCR *ttr*, Monoplex -qPCR *InvA*, Multiplex -qPCR *ttr* and Multiplex -qPCR *InvA*. A summary of the characteristics of the test methods. Specific values are indicated for sensitivity and specificity. Estimates were made for time to assay result, person-labour time requirement, cost of the test, specialist equipment cost by ranking them from Low to very high. Comparisons were also made for follow-up tests, antibiotic sensitivity and serotyping, and efficiency in batching.

	Salmonella stool culture	Monoplex -qPCR *ttr*	Monoplex -qPCR *InvA*	Multiplex -qPCR *ttr*	Multiplex -qPCR *InvA*
Sensitivity	Moderate (62.88%)	Very high (99.53%)	Very High (95.06%)	High (90.30%)	High (89.41%)
Specificity	Very high (99.99%)	Very High (95.46%)	High (90.31%)	Very high (99.30%)	Very high (98.00%)
Estimated time to assay result (10 samples)	3 – 7 days	1.5 days including overnight Selenite F broth enrichment	1.5 days including overnight Selenite F broth enrichment	0.5 day	0.5 day
Person-labour time requirement	Very high	High	High	high	high
Cost estimate	Low	High	High	Very high	Very high
Specialist equipment cost	Low: can be done in a standard microbiology laboratory	High: qPCR machine	High: qPCR machine	High: TAC-compatible qPCR machine Compatible centrifuge buckets TAC plate sealer	High: TAC-compatible qPCR machine Compatible centrifuge Buckets TAC plate sealer
Follow-up antibiotic sensitivity	Possible	Not possible	Not possible	Not possible	Not possible
Serotyping	Possible	Possible using serotype-specific primers	Possible using serotype-specific primers	Possible using serotype-specific primers	Possible using serotype-specific primers
Efficiency in batching	Low: working on more samples at once reduces efficiency	Very High: batched samples can be efficiently extracted and tested 96 samples and controls can be tested using 1 plate	Very High: batched samples can be efficiently extracted and tested 96 samples and controls can be tested using 1 plate	Very High: batched samples can be efficiently extracted and tested 8 samples and multiple pathogens can be tested using 1 plate	Very High: batched samples can be efficiently extracted and tested 8 samples and multiple pathogens can be tested using 1 plate

TAC-*InvA*, *ttr*, and *InvA*, demonstrated high specificity and sensitivity rates. Compared to the other methods, the monoplex based qPCR *ttr* achieved, in our opinion, the best sensitivity-specificity trade-off as it demonstrates near-perfect sensitivity (99.53%) and still achieves high specificity (95.43%). While monoplex *ttr* has the best overall performance, depending on the research context or clinical purpose, practitioners may still prefer tests with higher specificity such as stool culture, TAC-*ttr*, or TAC-*InvA*. All molecular test methods had significantly higher sensitivities than stool culture. High specificity and low sensitivity rates for culture have been widely reported[18]. Such low sensitivity rates should be taken into consideration when evaluating diagnostic tests. It is clear that a reference test with poor sensitivity is not adequate to evaluate alternative test methods. In such a situation, alternative means of evaluating the assays should be used, such as the LMM that has been used here. LMMs, and their counterpart for cross-sectional data, latent class models (LCMs), have been used to evaluate diagnostic tests for various pathogens, including *Salmonella*[48].

PPV and NPV vary depending on the prevalence of the condition being tested in any particular population. Our samples were collected from a population that was considered healthy and asymptomatic at the time of recruitment. Using the model-estimated stationary probability of being infected, we estimated the Salmonella infection prevalence of 5.25% in this population. With this prevalence estimate, stool culture demonstrated a high PPV (99.73%) when compared to molecular tests that had high NPVs, with the highest NPV recorded by mono-*ttr* (99.67%). As a trade-off between PPV and NPV, in a population of asymptomatic children in urban Blantyre, TAC-*ttr* achieved high PPV (85.97%) and high NPV (90.33%). Whether a test with high PPV or NPV is to be preferred depends on the research and/or clinical context. When prevalence is low, a slight change in specificity will have significant effects on the PPV. Higher PPVs could be observed in a situation where prevalence is high such as when using a cohort of hospitalised diarrheal cases or during a diarrhoeal outbreak.

Molecular methods had higher sensitivity but lower specificity relative to stool culture. The loss in specificity is slight compared to the gain in sensitivity, and in the case of *Salmonella*, the public health cost of false-negative results could be higher if the infection becomes potentially life-threatening due to withholding or delay of treatment. With the high sensitivity, molecular methods were able to detect asymptomatic *Salmonella* events, critical for the research questions we hoped to pose in this cohort. All the events that were detected here were asymptomatic in healthy children, which are potentially very important in transmission or the development of immunity. The detection of low bacterial burden events could also be relevant in settings like Malawi where unprescribed over-the-counter antibiotic procurement and use is common. Studies that have reported on risk factors of having a culture-negative result have indicated that antibiotic usage before sample collection is the main risk factor. Using molecular techniques

such as PCR could overcome this challenge because it detects bacterial DNA regardless of pathogen viability. This might increase the probability of identifying the infection and reduce sample processing time, leading to proper patient management and treatment if needed.

Our study has several limitations. One main limitation is the use of different sample types for the two qPCR platforms. The use of selenite sub-cultured stool samples in monoplex qPCR may have contributed to the superior performance when compared with the multiplex qPCR. We used neat stool samples for multiplex qPCR to comply with the manufacturer's protocol. Other studies have, however, demonstrated that testing primer/probe sets in the monoplex platform perform better than in the multiplex qPCR platform. Clinical samples used to test the performance of the test are a limitation, especially in determining the PPV and NPV. Clinical samples used in the study were collected from a cohort of children that were asymptomatic to *Salmonella* and remained healthy for most of the one-year study period. Using samples from participants with a clinical diagnosis of Salmonella or diarrhoea would have resulted in PPV and NPV estimates more directly relevant in a clinical setting. Another important limitation is the model-based nature of our approach. This was a necessity given the lack of a gold standard diagnostic test but does mean that our estimates depend on the validity of the model's assumptions, in particular, i) the assumption that the latent infection state at a given time only depends on the immediate previous timepoint, the so-called Markov assumption, and ii) the conditional independence assumption of the basic LMM. While our modelling framework had been extended to relax the latter assumption, a better fit was achieved for the basic model.

Conclusion

The data presented here demonstrate that the addition of selenite F broth pre-enrichment step increases *Salmonella* detection in stool samples and that *ttr* and *InvA* primer and probe sets used can detect different Salmonella strains. The ability of *ttr* to detect *Salmonella* with such high levels of specificity and sensitivity when tested using clinical samples collected from a mostly healthy cohort makes it a promising assay that could be used for research surveillance studies. The assays could be very useful in studying the transmission of *Salmonella* infections. This method may perform with different sensitivity and specificity in a chronic carriage, diarrhoeal or invasive *Salmonella* disease state, since the load and culturability of the pathogen within the stool may be different, and further validation studies would be needed

We established that selenite pre-culture increased diagnostic yield for molecular detection and identified *ttr* primers as molecular tools that could best help reveal the true extent of *Salmonella* exposure events within the gastrointestinal tract. This will allow us to understand their importance to diarrhoeal and invasive disease pathogenesis and epidemiology in the future.

Data availability

Underlying data

Figshare: Data and software code for Bayesian mixed latent Markov models for binary diagnostic data, https://doi.org/10.6084/m9.figshare.12911870.

1. gitMarcH-Bayesian-mixed-latent-Markov-models-for-binary-diagnostic-data.zip (software code for Latent Markov Model used in this study)

2. Data files used by the uploaded software code:
   - salexpoLIMSDataSetComplete.csv (Date of sample collection and follow-up visit number)
   - TACResults_4Mar TAC *ttr* TAC *InvA* Ct For Correlation.csv (Ct values for TAC_*ttr* and TAC_*InvA*)
   - *ttr* & *InvA* master file Ct for correlation.csv (Ct values for monoplex *TTR* and *InvA*)
   - *TtrInvA*Sensitivity20170724_corrected.csv (Combined binary results for stool culture, *ttr*, *InvA*, TAC_*ttr*, and TAC_*InvA* used to calculate sensitivity, specificity and correction of the test methods)

3. Raw data:
   - TAC Results_TAC-*ttr*_TAC-*InvA*_I_Ct ValuesTAC Results_TAC-*ttr*_TAC-*InvA*_IC_Ct-values.csv (raw Taqman array card assay results for test and control sample)
   - Salmonella_Detection_Stool_*ttr*_*InvA*_raw_data.xlsx (raw data for the monoplex qPCR assay. Includes results for test and control sample)

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Acknowledgements

References

1. Crump JA: **Progress in Typhoid Fever Epidemiology.** *Clin Infect Dis.* 2019; **68**(Suppl 1): S4–s9.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

2. Ao TT, Feasey NA, Gordon MA, *et al.*: **Global burden of invasive nontyphoidal *Salmonella* disease, 2010(1).** *Emerg Infect Dis.* 2015; **21**(6): 941–949.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

3. GBD 2017 Typhoid and Paratyphoid Collaborators: **The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017.** *Lancet Infect Dis.* 2019; **19**(4): 369–381.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

4. GBD 2017 Non-Typhoidal Salmonella Invasive Disease Collaborators: **The global burden of non-typhoidal salmonella invasive disease: a systematic analysis for the Global Burden of Disease Study 2017.** *Lancet Infect Dis.* 2019; **19**(12): 1312–1324.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

5. Crump JA, Luby SP, Mintz ED: **The global burden of typhoid fever.** *Bull World Health Organ.* 2004; **82**(5): 346–53.
   PubMed Abstract | **Free Full Text**

6. WHO: ***Salmonella* (non-typhoidal). Fact sheets -detail**. 2018 [cited 2019 October 4].
   Reference Source

7. GBD 2017 Non-Typhoidal Salmonella Invasive Disease Collaborators: **The global burden of non-typhoidal *salmonella* invasive disease: a systematic analysis for the Global Burden of Disease Study 2017.** *Lancet Infect Dis.* 2019; **19**(12): 1312–1324.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

8. Gordon MA: ***Salmonella* infections in immunocompromised adults.** *J Infect.* 2008; **56**(6): 413–422.
   PubMed Abstract | **Publisher Full Text**

9. Feasey NA, Dougan G, Kingsley RA, *et al.*: **Invasive non-typhoidal *salmonella* disease: an emerging and neglected tropical disease in Africa.** *Lancet.* 2012; **379**(9835): 2489–2499.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

10. Nyirenda TS, Gilchrist JJ, Feasey NA, *et al.*: **Sequential acquisition of T cells and antibodies to nontyphoidal *Salmonella* in Malawian children.** *J Infect Dis.* 2014; **210**(1): 56–64.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

11. MacLennan CA, Gondwe EN, Msefula CL, *et al.*: **The neglected role of antibody in protection against bacteremia caused by nontyphoidal strains of *Salmonella* in African children.** *J Clin Invest.* 2008; **118**(4): 1553–1562.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

12. Kotloff KL: **The Burden and Etiology of Diarrheal Illness in Developing Countries.** *Pediatr Clin North Am.* 2017; **64**(4): 799–814.
    PubMed Abstract | **Publisher Full Text**

13. Darton TC, Zhou L, Blohmke CJ, *et al.*: **Blood culture-PCR to optimise typhoid fever diagnosis after controlled human infection identifies frequent asymptomatic cases and evidence of primary bacteraemia.** *J Infect.* 2017; **74**(4): 358–366.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

14. Mogasale V, Ramani E, Mogasale VV, *et al.*: **What proportion of *Salmonella* Typhi cases are detected by blood culture? A systematic literature review.** *Ann Clin Microbiol Antimicrob.* 2016; **15**(1): 32.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

15. Antillon M, Saad NJ, Baker S, *et al.*: **The Relationship Between Blood Sample Volume and Diagnostic Sensitivity of Blood Culture for Typhoid and Paratyphoid Fever: A Systematic Review and Meta-Analysis.** *J Infect Dis.* 2018; **218**(suppl_4): S255–S267.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

16. Tennant SM, Toema D, Qamar F, *et al.*: **Detection of Typhoidal and Paratyphoidal *Salmonella* in Blood by Real-time Polymerase Chain Reaction.** *Clin Infect Dis.* 2015; **61 Suppl 4**(Suppl 4): S241–50.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

17. Msefula CL, Olgemoeller F, Jambo N, *et al.*: **Ascertaining the burden of invasive *Salmonella* disease in hospitalised febrile children aged under four years in Blantyre, Malawi.** *PLoS Negl Trop Dis.* 2019; **13**(7): e0007539.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

18. Andrews JR, Ryan ET: **Diagnostics for invasive *Salmonella* infections: Current challenges and future directions.** *Vaccine.* 2015; **33 Suppl 3**(03): C8–15.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

19. Trafford G, Ratnaraja N, Wickramasinghe N: **Molecular diagnostic testing for common stool pathogens.** *J Hosp Infect.* 2015; **90**(3): 196–8.
    PubMed Abstract | **Publisher Full Text**

20. Nyirenda TS: **Natural Immunity to *Salmonella* in Humans**. In: *Institute of Infection and Global Health.* University of Liverpool. 2015.

21. Malorny B, Paccassoni E, Fach P, *et al.*: **Diagnostic real-time PCR for detection of *Salmonella* in food.** *Appl Environ Microbiol.* 2004; **70**(12): 7046–52.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

22. Malorny B, Hoorfar J, Bunge C, *et al.*: **Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard.** *Appl Environ Microbiol.* 2003; **69**(1): 290–6.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

23. MacLennan CA, Gondwe EN, Msefula CL, *et al.*: **The neglected role of antibody**

in protection against bacteremia caused by nontyphoidal strains of *Salmonella* in African children. *J Clin Invest.* 2008; **118**(4): 1553–1562.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

24. Kingsley RA, Msefula CL, Thomson NR, *et al.*: **Epidemic multiple drug resistant** *Salmonella* **Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype.** *Genome Res.* 2009; **19**(12): 2279–87.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

25. Lertsethtakarn P, Silapong S, Sakpaisal P, *et al.*: **Travelers' Diarrhea in Thailand: A Quantitative Analysis Using TaqMan® Array Card.** *Clin Infect Dis.* 2018; **67**(1): 120–127.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

26. Platts-Mills JA, Liu J, Rogawski ET, *et al.*: **Use of quantitative molecular diagnostic methods to assess the aetiology, burden, and clinical characteristics of diarrhoea in children in low-resource settings: a reanalysis of the MAL-ED cohort study.** *Lancet Glob Health.* 2018; **6**(12): e1309–e1318.
Publisher Full Text

27. Liu J, Kabir F, Manneh J, *et al.*: **Development and assessment of molecular diagnostic tests for 15 enteropathogens causing childhood diarrhoea: a multicentre study.** *Lancet Infect Dis.* 2014; **14**(8): 716–724.
PubMed Abstract | **Publisher Full Text**

28. Liu J, Gratz J, Amour C, *et al.*: **A laboratory-developed TaqMan Array Card for simultaneous detection of 19 enteropathogens.** *J Clin Microbiol.* 2013; **51**(2): 472–80.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

29. Bartolucci F, Farcomeni A, Pennoni F: **Latent Markov Models for Longitudinal Data**. Chapman & Hall, CRC Press, Boca Raton, FL. 2013.

30. Henrion MYR, Chirambo A, Nyirenda TS, *et al.*: **Mixed latent Markov models for longitudinal multiple diagnostics data with an application to** *salmonella* **in Malawi.** *JSM Proceedings Vancouver Canada.* 2018.
Reference Source

31. Plummer M: **rjags: Bayesian Graphical Models using MCMC**. R package version 4-6. 2016.
Reference Source

32. Koukounari A, Donnelly CA, Moustaki I, *et al.*: **A latent Markov modelling approach to the evaluation of circulating cathodic antigen strips for schistosomiasis diagnosis pre- and post-praziquantel treatment in Uganda.** *PLoS Comput Biol.* 2013; **9**(12): e1003402.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

33. Koukounari A, Moustaki I, Grassly NC, *et al.*: **Using a nonparametric multilevel latent Markov model to evaluate diagnostics for trachoma.** *Am J Epidemiol.* 2013; **177**(9): 913–922.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

34. Gelman SA, Rubin DB, Rubin DB: **Inference from Iterative Simulation Using Multiple Sequences.** *Stat Sci.* 1992; **7**: 457–472.
Publisher Full Text

35. Bakdash JZ, Marusich LR: **Repeated Measures Correlation.** *Front Psychol.* 2017; **8**: 456.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

36. Kuijpers LMF, Chung P, Peeters M, *et al.*: **Diagnostic accuracy of antigen-based immunochromatographic rapid diagnostic tests for the detection of** *Salmonella* **in blood culture broth.** *PLoS One.* 2018; **13**(3): e0194024.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

37. Bugarel M, den Bakker H, Grout J, *et al.*: **CRISPR-based assay for the**

molecular identification of highly prevalent *Salmonella* serotypes. *Food Microbiol.* 2018; **71**: 8–16.
PubMed Abstract | **Publisher Full Text**

38. Pouzol S, Tanmoy AM, Pouzol DA, *et al.*: **Clinical Evaluation of a Multiplex PCR for the Detection of** *Salmonella enterica* **Serovars Typhi and Paratyphi A from Blood Specimens in a High-Endemic Setting.** *Am J Trop Med Hyg.* 2019; **101**(3): 513–520.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

39. Heymans R, Vila A, van Heerwaarden CAM, *et al.*: **Rapid detection and differentiation of** *Salmonella* **species,** *Salmonella* **Typhimurium and** *Salmonella* **Enteritidis by multiplex quantitative PCR.** *PLoS One.* 2018; **13**(10): e0206316.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

40. Scopes E, Screen J, Evans K, *et al.*: **Evaluation of the Thermo Scientific RapidFinder™** *Salmonella* **Species, Typhimurium, and Enteritidis Multiplex PCR Kit.** *J AOAC Int.* 2018; **101**(4): 1059–1100.
PubMed Abstract | **Publisher Full Text**

41. Park SH, Ricke SC: **Development of multiplex PCR assay for simultaneous detection of** *Salmonella* **genus,** *Salmonella* **subspecies I, Salm. Enteritidis, Salm. Heidelberg and Salm. Typhimurium.** *J Appl Microbiol.* 2015; **118**(1): 152–60.
PubMed Abstract | **Publisher Full Text**

42. Hannet I, Engsbro AL, Pareja J, *et al.*: **Multicenter evaluation of the new QIAstat Gastrointestinal Panel for the rapid syndromic testing of acute gastroenteritis.** *Eur J Clin Microbiol Infect Dis.* 2019; **38**(11): 2103–2112.
PubMed Abstract | **Publisher Full Text**

43. Martín A, Pérez-Ayala A, Chaves F, *et al.*: **Evaluation of the multiplex PCR Allplex-GI assay in the detection of bacterial pathogens in diarrheic stool samples.** *J Microbiol Methods.* 2018; **144**: 33–36.
PubMed Abstract | **Publisher Full Text**

44. Beckman AK, Ferrieri P: **Prospective Investigation of an Automated PCR/Nucleic Acid Microarray-Based Platform for Enteric Pathogen Testing.** *Lab Med.* 2019; **50**(4): 390–395.
PubMed Abstract | **Publisher Full Text**

45. Gilchrist JJ, MacLennan CA: **Invasive Nontyphoidal** *Salmonella* **Disease in Africa.** *EcoSal Plus.* 2019; **8**(2).
PubMed Abstract | **Publisher Full Text**

46. Boer MD, de Boer RF, Lameijer A, *et al.*: **Selenite enrichment broth to improve the sensitivity in molecular diagnostics of** *Salmonella*. *J Microbiol Methods.* 2018; **157**: 59–64.
PubMed Abstract | **Publisher Full Text**

47. Goneau LW, Mazzulli A, Trimi X, *et al.*: **Evaluating the preservation and isolation of stool pathogens using the COPAN FecalSwab™ Transport System and Walk-Away Specimen Processor.** *Diagn Microbiol Infect Dis.* 2019; **94**(1): 15–21.
PubMed Abstract | **Publisher Full Text**

48. Moore CE, Pan-Ngum W, Wijedoru LPM, *et al.*: **Evaluation of the diagnostic accuracy of a typhoid IgM flow assay for the diagnosis of typhoid fever in Cambodian children using a Bayesian latent class model assuming an imperfect gold standard.** *Am J Trop Med Hyg.* 2014; **90**(1): 114–120.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

49. Henrion MYR: **Data and software code for Bayesian mixed latent Markov models for binary diagnostic data.** *figshare.* Dataset. 2020.
http://www.doi.org/10.6084/m9.figshare.12911870.v3

Open Peer Review

Current Peer Review Status: ✔ ✔

- -

Version 2

Reviewer Report 06 May 2021

https://doi.org/10.21956/wellcomeopenres.18556.r43663

✔ **JY Zhang** (iD)

National Institute for Communicable Disease Control and Prevention, Beijing, China

The revised manuscript has improved a lot. Some details are not concise and accurate, but they provide new ideas for the evaluation of salmonella detection methods.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular detection of intestinal pathogens.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 05 May 2021

https://doi.org/10.21956/wellcomeopenres.18556.r43662

✔ **Qiuchun Li** (iD)

Key Laboratory of Prevention and Control of Biological Hazard Factors (Animal Origin) for Agri-food Safety and Quality, Ministry of Agriculture of China, Yangzhou University, Yangzhou, China

The manuscript has been revised seriously and is acceptable for indexing.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Prevalence of Salmonella in Foods and humans.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

- -

Version 1

Reviewer Report 27 January 2021

https://doi.org/10.21956/wellcomeopenres.17920.r41999

✗ **Qiuchun Li** [ID]

Key Laboratory of Prevention and Control of Biological Hazard Factors (Animal Origin) for Agri-food Safety and Quality, Ministry of Agriculture of China, Yangzhou University, Yangzhou, China

This study used four different methods (monoplex- qPCR TTR, monoplex- qPCR invA, multiplex TAC-TTR, multiplex TAC InvA) in detecting *Salmonella* in stool samples with comparison to the traditional stool culture methods. The manuscript is not well-written and hard to follow. I have some major questions:
1. Application of these methods into 421 stool samples, 40, 29, 56, and 47 samples were detected positive using mono-TTR, mono-InvA, TAC-TTR, and TAC-InvA, respectively. While 23 samples were detected positive using the stool culture method. the study concluded the detection methods using TTR primers could be used for research surveillance studies, but didn't confirm which method is reliable and applicable? And why?

2. When using the mon-TTR and mono-InvA, the genomic DNA used is from the selenite broth cultured samples, which is time-consuming for cultivation of the stool samples, so what is the advantage of these methods?

3. The specificity of the molecular methods used only 9 *Salmonella* serotypes, why not use some frequently reported human nontyphoidal *Salmonella* serotypes like *S.* London, *S.* Rissen, and *S.* Meleargidis, *S.* senftenberg, etc.? In addition, it is better to give the background of the prevalence of TTR and InvA in *Salmonella* serotypes based on genome sequencing analysis. The TAC is also used multiplex PCR methods, there is no detailed information on TAC in the manuscript.

4. The PPV and NPV have also been used to evaluate the molecular detection methods, under the condition of an estimated 5.25% of *Salmonella* in asymptomatic people, which method is better based on analysis of PPV and NPV?

5. It is necessary to revise the English grammar of the manuscript.

Is the rationale for developing the new method (or application) clearly explained?

Partly

Is the description of the method technically sound?

Partly

Are sufficient details provided to allow replication of the method development and its use by others?

Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Prevalence of Salmonella in Foods and humans.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 12 Apr 2021
Angeziwa Chirambo, Malawi-Liverpool-Wellcome (MLW) Programme, Blantyre, Malawi

Reviewer Report 2
27 Jan 2021 | for Version 1
Reviewer: Qiuchun Li
Affiliation: Key Laboratory of Prevention and Control of Biological Hazard Factors (Animal Origin) for Agri-food Safety and Quality, Ministry of Agriculture of China, Yangzhou University, Yangzhou, China

Outcome: Not Approved
Reviewer general comment:
This study used four different methods (monoplex- qPCR TTR, monoplex- qPCR invA, multiplex TAC-TTR, multiplex TAC InvA) in detecting *Salmonella* in stool samples with comparison to the traditional stool culture methods. The manuscript is not well-written and hard to follow. I have some major questions:

Author's general response
More effort has been made to make the manuscript clear by carefully proof-reading of the manuscript and correcting any grammatical errors and have reviewed some of the scientific

content as detailed in the below responses.

Reviewer comment 1:
 Application of these methods into 421 stool samples, 40, 29, 56, and 47 samples were detected positive using mono-TTR, mono-InvA, TAC-TTR, and TAC-InvA, respectively. While 23 samples were detected positive using the stool culture method. The study concluded the detection methods using TTR primers could be used for research surveillance studies but didn't confirm which method is reliable and applicable? And why?

Author's response 1:
We thank the reviewer for highlighting that the main conclusion was not stated clearly enough. Mono-TTR is the method which, based on our data and analysis approach, achieves, in our opinion, the best trade-off between sensitivity and specificity. However, depending on context and purpose, other researchers and practitioners may prefer to prioritise sensitivity or specificity differently and therefore prefer one of the other methods we discussed.

We have clarified this in the Results and Discussion sections. In the Results section, we now state:

"While stool culture achieved the highest specificity and monoplex TTR the highest sensitivity, monoplex TTR achieved arguably the best trade-off between sensitivity-and specificity: very high sensitivity (99.53%) at a relatively small drop in specificity (95.46) compared to stool culture."
In the Discussion section, we now clarify:

"Compared to the other methods, the monoplex based qPCR TTR achieved, in our opinion, the best sensitivity-specificity trade-off as it demonstrates near-perfect sensitivity (99.53%) and still achieves high specificity (95.43%). While monoplex TTR has the best overall performance, depending on the research context or clinical purpose, practitioners in different contexts may still choose tests with higher specificity such as stool culture, TAC-TTR or TAC-InvA".
As per our response to reviewer 1, point 3, we also more clearly stated that our test performance characteristics have been derived using a statistical model. In Methods, we now state:

"Another important limitation is the model-based nature of our approach. This was a necessity given the lack of a gold standard diagnostic test but does mean that our estimates depend on the validity of the model's assumptions, in particular i) the assumption that the latent infection state at a given time only depends on the immediate previous timepoint, the so-called Markov assumption, and ii) the conditional independence assumption of the basic LMM. While our modelling framework had been extended to relax the latter assumption, the better fit was achieved for the basic model."

Reviewer comment 2:
When using the mono-TTR and mono-InvA, the genomic DNA used is from the selenite broth cultured samples, which is time-consuming for cultivation of the stool samples, so what is the advantage of these methods?

Author's response 2:
A table showing the characteristics of *Salmonella* stool culture, Monoplex -qPCR *ttr,* Monoplex -qPCR *InvA,* Multiplex -qPCR *ttr and* Multiplex -qPCR *InvA* has been added to summarise the pros and cons of the test methods (Table 3).
A table has been added to summarise the characteristics of the different salmonella detection methods used in this study in order to highlight the strengths and weaknesses of

the methods used.

Reviewer comment 3:
The specificity of the molecular methods used only 9 *Salmonella* serotypes, why not use some frequently reported human nontyphoidal *Salmonella* serotypes like *S.* London, *S.* Rissen, and *S.* Meleargidis, *S.* senftenberg, etc.? In addition, it is better to give the background of the prevalence of TTR and InvA in *Salmonella* serotypes based on genome sequencing analysis.

<u>***Author's response 3:***</u>
The following additions have been made to the Methods section subsection "Monoplex-qPCR ttr and InvA assay"
"The use of ttr was based on (Malorny et al., 2004). In this study, the specificity of the ttr gene was assessed using 110 Salmonella strains representing 31 serotypes, and it demonstrated 100% specificity. The InvA gene has been widely studied and used as a pan Salmonella marker. In (Malorny, Hoorfar, Bunge, & Helmuth, 2003), the specificity of InvA gene was assessed using 242 Salmonella strains representing 43 serotypes. It also demonstrated 100% specificity. InvA has shown 100% specificity in several other studies."
The following additions have been made to the Methods section, subsection "Specificity of ttr and InvA primer/probe set for Salmonella compared to other local pathogens"
"To determine the prevalence of ttr and InvA in the genomes of Salmonella serotypes, NCBI nucleotide blast was conducted (10 Feb 2021). Ttr and InvA nucleotide sequences were used as the query sequence against Salmonella enterica subspecies enterica (taxid:59201) genomes with a maximum target sequence set at 1000. Both primer sequences demonstrated 100% identity for most of the commonly isolated Salmonella serotypes."
"For the invtro work, All Salmonella isolates with known serotypes available in the MLW blood culture isolate archive were used."
Regarding the reviewers comments on TAC methodology, more details on TAC has been added to the manuscript (Methods section, subsection "Detection of Salmonella using multiplex qPCR assay
" The performance of the TAC method has been previously described and has now been widely used. It is used to detect multiple enteric pathogens, including bacteria, viruses, protozoa, and helminths. Targets included on the TAC card for pan Salmonella detection are InvA and ttr. Phocine Herpesvirus (PHhv) and MS2 targets are included as internal positive controls. "

Reviewer comment 4:
 The PPV and NPV have also been used to evaluate the molecular detection methods, under the condition of an estimated 5.25% of *Salmonella* in asymptomatic people, which method is better based on analysis of PPV and NPV?

<u>***Author's response 4:***</u>
PPV and NPV results are stated in Table 3 and Figure 1B. However, we feel it is more useful to compare the different methods using sensitivity and specificity. These are direct characteristics of the diagnostic tests whereas PPV and NPV depend on the prevalence of the condition being assessed in the population that is being studied. As such, our results for PPV and NPV are applicable in the context of an asymptomatic (at recruitment) population of infants in urban Blantyre, Malawi and are not immediately generalisable to other contexts. This said, according to Table 3 and Figure 1B, stool culture achieves the best PPV and mono-TTR the best NPV (in both cases virtually 100%). Overall best trade-off is probably

achieved by TAC-TTR achieving both high PPV and NPV– but again it depends on the purpose of the researcher / clinician whether to prioritise high PPV or high NPV or something in-between. We have clarified this in the manuscript. In Results we now state:
"While stool culture has highest PPV (99.73%) and mono-TTR the largest NPV (99.67%), arguably the best PPV and NPV trade-off is achieved by TAC-TTR (both high PPV, 85.97%, and high NPV, 90.33%). We note that these PPV and NPV estimates are for an asymptomatic population of children in urban Blantyre, Malawi and are not directly generalisable to different contexts and populations where prevalence may be higher or lower."
And in the Discussion section we now state:
"PPV and NPV vary depending on the prevalence of the condition being tested in any particular population. Our samples were collected from a population that was considered healthy and asymptomatic at the time of recruitment. Using the model-estimated stationary probability of being infected, we estimated the Salmonella infection prevalence of 5.25% in this population. With this prevalence estimate, stool culture demonstrated a high PPV (99.73%) when compared to molecular tests that had high NPVs, with the highest NPV recorded by mono-TTR (99.67%). As a trade-off between PPV and NPV, in a population of asymptomatic children in urban Blantyre, TAC-TTR achieved both high PPV (85.97%) and high NPV (90.33%). Whether a test with high PPV or NPV is to be preferred depends on the research and/or clinical context."

Reviewer comment: It is necessary to revise the English grammar of the manuscript.
Author's response: We have done a careful proof-reading of the manuscript and corrected any grammatical errors.
   ○ Is the rationale for developing the new method (or application) clearly explained?

Reviewer comment: Partly
Author's response: We trust that the amendments made now fully describe the rationale.
   ○ Is the description of the method technically sound?

Reviewer comment: Partly
Author's response: We trust that the amendments made now give a complete technical description.
   ○ Are sufficient details provided to allow replication of the method development and its use by others?
Reviewer comment: Partly
Author's response: We trust that the amendments made now mean this method could be fully replicated.
   ○ If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Reviewer comment: Partly
Author's response: The reviewer makes no specific comment above relating to this. We believe that in keeping with Reviewer 1's opinion, all data are available to make the results reproducible.
   ○ Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Reviewer comment: Partly
Author's response: We trust that the amendments made above mean that the conclusions are fully supported by the findings.

Competing Interests
No competing interests were disclosed.

Reviewer Expertise
Prevalence of Salmonella in Foods and humans.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Henrion, M. Y. R., Chirambo, A., Nyirenda, T.S., Gordon, M. (2018). Mixed latent Markov models for longitudinal multiple diagnostics data with an application to Salmonella in Malawi. *JSM Proceedings Vancouver, Canada,*.
Malorny, B., Hoorfar, J., Bunge, C., & Helmuth, R. (2003). Multicenter validation of the analytical accuracy of Salmonella PCR: towards an international standard. *Appl Environ Microbiol, 69*(1), 290-296. doi:10.1128/aem.69.1.290-296.2003
Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A., & Helmuth, R. (2004). Diagnostic real-time PCR for detection of Salmonella in food. *Appl Environ Microbiol, 70*(12), 7046-7052. doi:10.1128/aem.70.12.7046-7052.2004

Competing Interests: No competing interests were disclosed.

Reviewer Report 21 January 2021

https://doi.org/10.21956/wellcomeopenres.17920.r41992

?   **JY Zhang** (iD)

National Institute for Communicable Disease Control and Prevention, Beijing, China

The authors optimized an in-house monoplex real-time PCR for the detection of *Salmonella ttr* and *invA* genes in stool, validated their specificity, and assessed their performance using clinical stool specimens collected from a cohort of 50 asymptomatic healthy children. The main problems:

1. Nucleic acid detection of enrichment culture can obtain the highest sensitivity, and the sensitivity of culture method is poor, which many studies have mentioned. The authors need to summarize the innovation points again to make it more attractive.

2. Unlike other studies, this study used continuously collected specimens from cohort. If the authors can analyze the queue data, it will be more meaningful.

3. When calculating the sensitivity and specificity of a method, it is necessary to determine whether the tested object is true positive or true negative. Whether the specimen is really positive can be determined by test. The author's conclusion was based on statistical analysis rather than experimental results, which I don't think is ideal.

Minor:
- Introduction: Gene names should be in italics and lowercase.

- Methods:

  - Salmonella stool culture: The volume of selenite F broth should be given. Did this method follow a literature or is it used in the authors' laboratory? Inoculating the centrifuged pellet onto XLD, I think there are too many bacteria, which may affect the subsequent selection of suspected colonies.

  - Table 1: The fluorescent groups and quenching groups of the probes needed to be given.

  - Statistical analysis: I don't understand the method used by the authors, so I don't comment on it. I suggest that the authors should explain the reliability of this method.

- Results and discussion. The analysis of cohort data is more meaningful. The reasons for the inconsistent results should be analyzed.

Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular detection of intestinal pathogens. I'm not familiar with the

statistical method mentioned by the author, so I suggest other reviewers review it again.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 12 Apr 2021
Angeziwa Chirambo, Malawi-Liverpool-Wellcome (MLW) Programme, Blantyre, Malawi

Reviewer report 1
Reviewer: JY Zhang
Affiliation: National Institute for Communicable Disease Control and Prevention, Beijing, China

21 Jan 2021 | for Version 1

Outcome: Approved with reservations

***Reviewer 1 comment*:**
The authors optimised an in-house monoplex real-time PCR for the detection of *Salmonella ttr* and *invA* genes in stool validated their specificity and assessed their performance using clinical stool specimens collected from a cohort of 50 asymptomatic healthy children. The main problems:

***Reviewer comment 1*:**
Nucleic acid detection of enrichment culture can obtain the highest sensitivity, and the sensitivity of the method is poor, which many studies have mentioned. The authors need to summarise the innovation points again to make it more attractive.
Authors' response 1:
A table showing the characteristics of *Salmonella* stool culture, Monoplex -qPCR *ttr,* Monoplex -qPCR *InvA,* Multiplex -qPCR *ttr, and* Multiplex -qPCR *InvA* has been added to summarise the pros and cons of the test methods (Table 3).
The following revision has been made to the discussion section of the manuscript
"By contrast, the advantage of a monoplex test is that it is economical than the multiplex, faster than stool culture, even with the addition of the selenite enrichment step and allows for batch-processing of samples which increases the efficiency of the test method."

***Reviewer comment 2*:**
Unlike other studies, this study used continuously collected specimens from cohorts. If the authors can analyse the queue data, it will be more meaningful.
Authors' response 2:
We are not sure that we fully understand what the reviewer means by 'queue' data. We take it to mean that the reviewer has questions around our use of longitudinal data. The statistical model (Henrion, 2018) has been developed to take account of the longitudinal nature of the data, with repeated observations of the same individuals. The model explicitly models the progression over time of the underlying infection process. This is a statistically

principled approach. The cohort data allows us to detect Salmonella exposure events as they happened – useful given that Salmonella prevalence can vary across the year.
If we have misunderstood the reviewer's comment, we are happy to further elaborate on the nature of the data and our analysis approach.

***Reviewer comment 3*:**
When calculating the sensitivity and specificity of a method, it is necessary to determine whether the tested object is true positive or true negative. Whether the specimen is really positive can be determined by test. The author's conclusion was based on statistical analysis rather than experimental results, which I don't think is ideal.

<u>***Authors' response 3*:**</u>
We thank the reviewer for highlighting that we were not clear enough about how we estimated the test performances. The reviewer is right to point out that we used a statistical model for assessing the various diagnostic methods. If a true, perfect gold standard method existed, then it would, of course be preferable to compare the methods against this perfect diagnostic test. However, in our case the only available reference standard, culture, is known to have low sensitivity. For this reason, we decided to use the statistical model described in the manuscript and also, in more detail, in a separate publication (Henrion, 2018)
The statistical model we used is of a particular type, specifically it is a latent Markov model (LMM), an accepted approach to model the underlying true infection state of individuals. The model uses this modelled latent infection state to estimate the sensitivity and specificity parameters that are most likely to have given rise to the observed data. As with any statistical model, there are a number of assumptions that have been made (in particular the LMM used in the present manuscript assumes i) that the infection state at a given timepoint only depends on the immediate prior timepoint and ii) that conditional on the latent state, the response probabilities of the different diagnostic tests are independent) and if these are not met, the results could be invalid.
Despite the need to use a statistical rather than an experimental approach, we have done some experimental validation. using spiked stool samples to assess detection limits for the molecular methods. We could not, however, use such spiked samples to calculate sensitivities and specificities: such artificial samples do not reflect true, biological samples and could lead to estimates for sensitivity and specificity that are biased to incorrectly high values. For this reason, we preferred to use real world data and a statistical approach to best estimate the test characteristics. Please note that this is a well-recognised and validated approach in this situation, and similar models to ours have been used by other authors to evaluate novel diagnostic tests (Koukpunari A, 2013)

We have made the following revisions to the manuscript to incorporate and clarify the above points:
  - In the Discussion section where we discuss the specificities and sensitivities that we estimated, we now clarify this is a model-based approach:

"Given the lack of a true gold standard diagnostic test, we took a model-based approach and used an LMM to estimate the specificities and sensitivities of the 5 Salmonella detection methods."

  - In the Discussion section, we now list the model-based approach as another limitation, given that our results depend on the validity of the model assumptions.

We now state:

"Another important limitation is the model-based nature of our approach. This was a necessity given the lack of a gold standard diagnostic test but does mean that our estimates depend on the validity of the model's assumptions, in particular) the assumption that the latent infection state at a given time only depends on the immediate previous timepoint, the so-called Markov assumption, and ii) the conditional independence assumption of the basic LMM. While our modelling framework had been extended to relax the latter assumption, the better fit was achieved for the basic model."

Minor:

Reviewer's comment: Introduction: Gene names should be in italics and lowercase.
Authors' response: Gene names have been changed to italics and lowercase

Reviewer's comment: Methods:
Salmonella stool culture: The volume of selenite F broth should be given. Did this method follow a literature or is it used in the authors' laboratory? Inoculating the centrifuged pellet onto XLD, I think there are too many bacteria, which may affect the subsequent selection of suspected colonies.
Authors' response:
The following changes have been made in the methods section under the subsection "Salmonella stool culture"

1. *"10 mls of selenite F broth was used. THis is a method that has been developed in our own laboratory."*
2. *"1 ml loop was used to subculture Salmonella from the pellet in order to achieve single colonies."*

Reviewer's comment:
Table 1: The fluorescent groups and quenching groups of the probes needed to be given.
Author's response:
The fluorescent and quenching groups have been added to the sequences for probes

Reviewer's comment:
Statistical analysis: I don't understand the method used by the authors, so I don't comment on it. I suggest that the authors should explain the reliability of this method.
Author's response:
We thank the reviewer for highlighting the difficulty to understand the model approached used. We will attempt to clarify the main ideas behind the modelling framework in this response. The necessity for a model-based approach is due to the fact that there is no good reference standard diagnostic method available. While our modelling framework allows for different complexity of data, the basic LMM used in this manuscript consists of two parts: i) a latent or unobserved process model for the underlying infection status, ii) a measurement model for the observed test results from the 5 diagnostic methods used. The process model estimates where a given study participant is infected or not and how the transitions between infected and non-infected progress over time. The measurement model estimates the probabilities of recording a positive, respectively a negative result, given whether or not the study participant is infected. These probabilities from the measurement model are the test sensitivities (probability of recording a positive result when infected) and specificities (probability of recording a negative result when not infected).

LMMs, and the related latent class analysis models for cross-sectional data, have been used extensively for evaluating novel diagnostic methods (e.g. (Koukpunari A, 2013; van Smeden, Naaktgeboren, Reitsma, Moons, & de Groot, 2014) With the caveat that results are only as valid as the model's assumptions, such models provide a reliable evaluation of diagnostic methods when there is no true gold standard. We have previously evaluated, using simulated data where the true infection state is known, the reliability of the LMM modelling framework used in the present manuscript (Henrion, 2018).

While we already refer to a separate paper describing the details of the model (Henrion, 2018), we have added the following clarification in the Methods section:

"LMMs consist of a process model for a latent condition (in our case the unobserved true infection status) evolving over time and a measurement model for the observed outputs (in our case the results from the 5 diagnostic test methods) conditional on the latent state".

Results and discussion.

Reviewer's comment:
The analysis of cohort data is more meaningful. The reasons for the inconsistent results should be analysed.

Author's response:
This point is not very clear to us. We would be happy to address this comment if the reviewer would shed more light.
- Is the rationale for developing the new method (or application) clearly explained?

Yes
- Is the description of the method technically sound?

Yes
- Are sufficient details provided to allow replication of the method development and its use by others?

Reviewer's comment: Partly

Author's response: Additional technical information has been added as requested
- If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes
- Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Reviewer's comment: Partly

Author's response:
We trust that the clarifications requested and made in the manuscript now adequately support the conclusions

Competing Interests
No competing interests were disclosed.

Reviewer Expertise
Molecular detection of intestinal pathogens. I'm not familiar with the statistical method

mentioned by the author, so I suggest other reviewers review it again.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Henrion, M. Y. R., Chirambo, A., Nyirenda, T.S., Gordon, M. (2018). Mixed latent Markov models for longitudinal multiple diagnostics data with an application to salmonella in Malawi. *JSM Proceedings Vancouver, Canada,*.

Koukpunari A, D., C. A., ..., et al., . (2013). A latent Markov modelling approach to the evaluation of circulating cathodic antigen strips for schistosomiasis diagnosis pre- and post-praziquantel treatment in Uganda. *PLOS Computational Biology,*.

van Smeden, M., Naaktgeboren, C. A., Reitsma, J. B., Moons, K. G., & de Groot, J. A. (2014). Latent class models in diagnostic studies when there is no reference standard--a systematic review. *Am J Epidemiol, 179*(4), 423-431. doi:10.1093/aje/kwt286

Competing Interests: No competing interests were disclosed.