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Evaluation of a *Salmonella* Strain Isolated from Honeybee Gut as a Potential Live Oral Vaccine Against Lethal Infection of *Salmonella* Typhimurium

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Abstract

In this research, *Salmonella* species were isolated from the animal, insect and human enteric sources in Faisalabad, Punjab, Pakistan. These species were characterized by different microbiological and molecular techniques including polymerase chain reaction (PCR) by amplification of the 16S rRNA gene. Furthermore, sequencing of the amplicons confirmed all ten isolates as *Salmonella* strains. The antigenic cross-reactivity was found maximum between the HB1 (strain isolated from honeybee) antiserum and its antigen with an antibody titer of 1:128, while the HB1 antiserum showed a cross-reactive titer range of 1:8 to 1:64. On the basis of the highest geometric mean titer (GMT) shown by the antiserum of the HB1 antigen, it was selected as the best candidate for a cross-reactive live *Salmonella* oral antigen. Moreover, the HB1 antigen was used a live oral antigen (1×10^{10} CFU/ml) in a safety test in rabbits and proved to be avirulent. During the animal trial, three different oral doses of the HB1 live oral antigen were evaluated in four different rabbits' groups (R1, R2, R3, and R4). The dose number 2 of 0.5 ml (two drops orally and repeated after one week) gave the best GMT measured by indirect hemagglutination (IHA) as compared to the other two doses, while R4 group was kept as control. Results of the challenge protection test also validated the efficacy of the double dose of the HB1 live vaccine, which gave the highest survival percentage. Results of this study lay the foundation for a potential cross-reactive live oral *Salmonella* vaccine that has proved to be immunogenic in rabbits.

Key words: *Salmonella*, antigen, cross-reactivity, 16S rRNA gene, geometric mean titer

Introduction

Globally, every year millions of humans and animals become victims of *Salmonella*-related infections. Both developed and underdeveloped countries are equally affected by these infections, and each year millions of dollars are exhausted on epidemiological studies, prevention, and treatment (Eng et al. 2015). In spite of advancements and improvements in hygiene and sanitation in many countries, *Salmonella*-related infections are still on the rise (Coburn et al. 2007; Crump et al. 2015). The most common manifestation of *Salmonella* infection is gastroenteritis followed by enteric fever and bacteremia (Majovicz et al. 2010; Medalla et al. 2017)

The genus *Salmonella* is a rod-shaped, Gram-negative, facultative anaerobe and a member of the family Enterobacteriaceae, it has two species *Salmonella enterica* and S*almonella bongori* (Kidgell et al. 2002; Coburn et al. 2007); for a review about the taxonomy of the genus *Salmonella* see Brenner et al. (2000). Division of *Salmonella* subspecies into serotypes was done on the basis of lipopolysaccharide, flagellar and carbohydrate structures (Naier et al. 2014). Both humans and animals can acquire the *S. enterica* serovars orally (McClelland et al. 2001). However, the manifestation of disease caused by these pathogens depends upon various factors such as host susceptibility, immune status of the host and the pathogenic serovar involved (Hauser et al. 2010).

The four major disease syndromes caused by *S. enterica* include diarrhea/enterocolitis, typhoid fever, bacteremia and chronic asymptomatic carriage (Bhan et al. 2005; Karon et al. 2007; Crump et al. 2015). The primary route of spread of *S*. Typhi in humans is the fecal-oral route. According to the latest World Health Organization (WHO) data from 2018, each year 11–20 million

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people get sick from typhoid fever, and between 128 000 to 161 000 people die due to the disease (WHO 2018). More than 90% of enteric fever cases occur in Asia alone (Crump et al. 2004), with Pakistan, Bangladesh, and Nepal being the most affected (Brooks et al. 2005; Karkey et al. 2013). If a suitable treatment regime is not adopted for the typhoid patients there is a 12–30% increase in the mortality, while in treated cases the survival rate can be up to 99% (Ochiai et al. 2008). Over the years, antimicrobial resistance in *Salmonella* serotypes has emerged as a serious health problem worldwide (Chiu et al. 2002). For more insight about antimicrobial resistance in *Salmonella* see Threlfall (2002), Foley et al. (2008), Kariuki et al. (2015).

Consumption of food or fluids contaminated with *Salmonella* result in infection in both humans and animals (Escobedo et al. 2011). After entry into the intestine, the bacteria attach, invade, and proliferate in the enterocytes of the gut-associated lymphoid tissue (GALT) (Monack et al. 2004). This can result in a diseased or carrier state and can also stimulate the induction of various immune responses (mucosal and systemic). However, these immune responses can be suppressed with avirulent invasive *Salmonella*, which can facilitate the establishment of a carrier state (Curtiss et al. 1993; Burton et al. 2014). It is possible to attenuate *Salmonella* strains by the introduction of various mutations. The main purpose of oral immunization of animals with avirulent *Salmonella* serotypes is usually not associated with suppression, but with the stimulation of cellular and humoral immune responses (Coburn et al. 2007). Animals injected with killed vaccines or bacterins exhibit short-lived humoral immunity, and this also does not stimulate the induction of mucosal and cellular immune response. Moreover, live *Salmonella* oral vaccines are better suited as vaccine candidates as they provide long-lasting immunity (Behnsen et al. 2015).

In many countries licensed typhoid vaccines are being used against typhoid fever. However, systemic reactions have developed in around 25–40% of patients (Eng et al. 2015). The majority of recipients of the typhoid vaccine have been either children or young adults. The Ty21a is an oral attenuated *Salmonella* vaccine, but it has not been adopted by countries due to its high cost (Fraser et al. 2007). Another vaccine used in the prevention of typhoid fever is Vi capsular polysaccharide subunit vaccine, but it has not shown positive results in children under the age of two years (Yang et al. 2001). Recently, a conjugate vaccine using O-polysaccharide (OSP) of *S*. Typhi and *S*. Paratyphi A conjugated with Diphtheria toxoid (DT) was used in a vaccine trial in mice. The trials confirmed the immunogenic potential of the conjugate vaccine (Ali et al. 2014). In the case of animals including poultry, various *Salmonella* vaccines are also in practice worldwide (Desin et al. 2013; Gayet et al. 2017).

Salmonella Typhimurium is a non-typhoidal serovar, which infects a wide range of animal hosts and humans (Feasey et al. 2012). Despite various attempts, an appropriate licensed vaccine against *S*. Typhimuirum for human use has not yet been approved (Ortiz et al. 2014). However, various vaccines (live, attenuated, subunit) are under the process of development, these vaccines may have the ability to induce a long-term cross-protective prophylaxis against non-typhoidal *Salmonella* serotypes (Sanapala et al. 2017). The aim of this study was to search out a *Salmonella* species that could be used as a potential live cross-reactive antigen against infection of *S*. Typhimurium in a rabbit model and could have the potential to be used as a live vaccine for future experiments.

For this purpose, a mass scale isolation of *Salmonella* species from different enteric sources was done using various microbiological and molecular tools. Initially, the whole cell antigen of all the identified strains was used in an immunological assay to determine the cross-reactivity. The best cross-reactive antigen was also evaluated for its avirulent potential in animal trials. Finally, the live antigen was tested for cross-protection against lethal challenge by *S*. Typhimurium (ATCC 14028) in an animal trial.

Experimental

Materials and Methods

Sample collection. Between June and July of 2016, a total of 90 samples were randomly collected from various enteric sources. These stool and fecal samples were collected from various veterinary hospitals, poultry/livestock farms and hospital facilities (Allied hospital, District headquarter hospital, Faisalabad) in the district of Faisalabad, Punjab, Pakistan (Table I). In the case of insects, honeybees were selected for sampling, the droppings were not clearly visible by the naked eye, so the gut contents were taken as samples. The samples were collected through sterile cotton swabs in different collection tubes containing Cary Blair transport medium (Oxoid, UK). All the samples were transported immediately to Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan for further processing.

Isolation and identification of *Salmonella* isolates. For pre-enrichment, the samples were suspended in phosphate buffer water (PBW) with the necessary volume of medium used to make a 1/10 dilution of each sample. Tetrathionate broth (TTB) (Oxoid, UK) was used for the primary enrichment of the fecal/stool/droppings samples, with 1 g of the sample in 10 ml of

Table I
Details of collection of the fecal/stool/dropping samples along with location of the sampling.

Species	Sample size	Average weight (gm)	Location of the sample collection
Human	15	7	Allied Hospital, Faisalabad, Pakistan. District Headquarters Hospital, Faisalabad, Pakistan
Cat	15	5	Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan
Sheep	15	4	Livestock farm, University of Agriculture, Faisalabad, Pakistan Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan
Honey bee	15	0.2	Botanical garden, University of Agriculture, Faisalabad, Pakistan Department of Entomology, University of Agriculture, Faisalabad, Pakistan
Poultry birds	15	3	Poultry farms, University of Agriculture, Faisalabad, Pakistan
Horse	15	8	Brooke Hospital, University of Agriculture, Faisalabad, Pakistan

TTB and incubated at 37°C for 48 hours. After enrichment, the subculturing was performed on *Salmonella-Shigella* agar (SSA) (Oxoid, UK), and further confirmation was done on Bismuth Sulphite Agar (BSA) (Oxoid, UK). In the case of SSA, the Petri plates were incubated for 24 hours, and the BSA plates were incubated for 48 hours at 37°C (Andrews et al. 1998). Visual examination of the colonies in the growth media was performed. Presumptive *Salmonella* colonies were straw colored with black centers on SSA and black colored colonies on BSA.

For further biochemical characterization, a biochemical kit RapID™ ONE (Remel R8311006) (ThermoFisher Scientific, USA) was used. Prior to inoculation into the kit, the well-differentiated colonies were picked up by a sterilized inoculating loop from both SSA plates and BSA plates and were inoculated into the Remel inoculation fluid (2 ml). The turbidity of the fluid was matched with the McFarland standard scale and was equal to 2.0 McFarland standard. The fluid containing the growth was then transferred to the wells of the kit and incubated at 37°C for 4 hours. The Rapid one panel (kit) contains 18 reaction cavities that provide a total test score of 19. The cavities 15–17 required the addition of two reagents, 1) RapID One reagent (ρ-dimeth ylaminocinnamaldehyde), the enzymatic hydrolysis of the acrylamide substrate releases free β-naphtylamine which is detected by this reagent 2) RapID Indole reagent (ρ-dimethylaminocinnamaldehyde, hydrochloric acid, and demineralized water), detects formation of indole. Scores were evaluated using the following link http://www.remel.com/ERIC/Home.aspx. *Salmonella* Typhimuirum (ATCC 14028) was used as a control for the validation of kit results.

Molecular identification by amplification of 16S rRNA genes. The ten isolates that were confirmed as *Salmonella* species from the biochemical tests were further subjected to polymerase chain reaction (PCR) by amplifying the 16S rRNA gene and further sequencing of the amplicons. The DNA extraction was performed using Genomic Isolate II DNA extraction kit (Bio line, London, UK) by following the instructions provided in the user manual for cultured cells. The NANODROP 8000 spectrophotometer (Thermo Scientific, USA) was used for the quantification of the DNA concentration in the samples.

For the amplification of the 16S rRNA gene of the isolates, PCR was done with primers 27 F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492 R (5'-GGTTACCTTGTTACGACTT-3'). For PCR, the conditions of the thermal cycler were set at initial denaturation at 96°C for 3 min, followed by 32 amplification cycles of 30 s at 94°C, 30 s at 52°C and 1 min at 70°C, followed by a final extension at 70°C for 10 min (Sipos et al. 2007). For gel electrophoresis, a marker (kb ladder, University of California, San Diego, USA), of 4 kbp size was used. The amplicons were further purified using QIAquick Kit (Qiagen, Germany). The pure PCR products were placed in ice boxes for transport and were sent for sequencing to the biotechnology company TSINGKE, China.

Phylogenetic analysis. The sequences obtained of all ten *Salmonella* isolates were screened against the National Center of Biotechnology (NCBI) database using the Basic Local Alignment Search Tool (BLAST). After BLAST confirmed all ten sequences to be *Salmonella*; these sequences and two *S. bongori* strains (as out-groups) were used to construct a phylogenetic tree using the Molecular Evolutionary Genetic Analysis (MEGA X) software (Kumar et al. 2018)

Preparation of antigen for the raising of antiserum in rabbits. The purified colonies of *Salmonella* strains were cultured in tryptic soy broth for 48 hours at 37°C. Cultures were transferred to Eppendorf tubes and were centrifuged at 800 × g. The supernatant was discarded, and the pellet was resuspended in phosphate buffer saline (PBS). Using the McFarland standard, a standard of 0.5 McFarland was maintained. The antigens in the bacterial culture were inactivated by the addition of 0.3% formalin, and an equal volume of Montanide adjuvant ISA 206VQ (SEPPIC) was added in the suspension and was mixed gently. The final count of

Table II
Schedule of *Salmonella* antigen inoculation for the raising of antibodies against *Salmonella* strains.

Salmonella antigen	Day 1 = 1st shot (0.2 ml)		Day 7 = 1st booster (0.5 ml)		Day 14th = 2nd booster (0.5 ml)	
	HR1	C	HR1	C	HR1	C
TY1	0.2	0.2	0.5	0.5	0.5	0.5
TY2	0.2	0.2	0.5	0.5	0.5	0.5
TY3	0.2	0.2	0.5	0.5	0.5	0.5
HB1	0.2	0.2	0.5	0.5	0.5	0.5
HB11	0.2	0.2	0.5	0.5	0.5	0.5
SP6	0.2	0.2	0.5	0.5	0.5	0.5
CT7	0.2	0.2	0.5	0.5	0.5	0.5
PB2	0.2	0.2	0.5	0.5	0.5	0.5
PB9	0.2	0.2	0.5	0.5	0.5	0.5
HE13	0.2	0.2	0.5	0.5	0.5	0.5

HR1 = Group 1 of rabbits given antigen, C = control group given normal saline

the Montanide adjuvanted antigens was 1×10^{10} CFU/ml and they were preserved at 4°C.

Protocol for raising antiserum against *Salmonella* strains. For the raising of antisera, twenty specific pathogen free (SPF) Chinchilla rabbits having an average weight of 3 kg were selected. Following all the bioethical and biosafety measures as approved by the Institutional Biosafety Committee of UAF, the rabbits were reared at the animal house of the Institute of Microbiology, UAF. The rabbits were divided into two groups HR1 and HC, with each group having ten rabbits in it (Table II). On the first day, 0.2 ml of the prepared antigens were injected intraperitoneally into rabbits of the group HR1, while 0.2 ml of normal saline was injected into the rabbits of group HC as described in. On the 7th day a booster dose of 0.5 ml was given to the rabbits of HR1, while 0.5 ml of normal saline was given to group C. On the 14th day a repeat of the booster dose for the group HR1 was done with 0.5 ml being injected again, while the group C rabbits were injected with 0.5 ml normal saline. The antiserum raised against the whole cell antigens of the *Salmonella* strains was collected on the 21st day of the experiment.

Microtiter plate agglutination test. This modified assay test was performed to check the antibody titer levels resultant from the antiserum reacting with the different *Salmonella* antigens (Barsoum and Awad 1972). It was performed in a 96-well microtiter plate in a triplicate with average readings presented in the Results section. A volume of 50 µl of tryptic soy broth was layered in each of the wells of the microtiter plate, 50 µl of the antiserum raised from all of the strains was layered at the start of each well and two-fold dilutions were done until the last well. A volume of 50 µl of the antigens from all of the isolates was mixed in a sequence. The plate was incubated for 12 hours at 37°C. A smooth mat shape at the bottom of the well indicated a positive reaction, while a negative reaction was indicated by button formation of the antigen at the bottom of the wells. The last serum dilution where agglutination was visible was considered the agglutination endpoint. Agglutination antibody titers were measured as the inverse of the greatest dilution that still yielded agglutination. For the geometric mean titer (GMT), the arithmetic means of the logarithms of the last positive serum dilution were considered.

Safety studies for evaluation of HB1 live antigen. Preserved cultures of HB1 strain were refreshed in tryptone soy broth (TSB). Colony count was performed, and a separate final suspension of 10^{10} CFU/ml was prepared. The *Salmonella* antigen (HB1) was maintained to a 0.5 McFarland standard and kept in phosphate buffer saline (PBS) (pH 7.2). As a preservative, honey (2%) was added and no inactivation of the antigen was done. The safety studies were conducted according to the international standards of antigen evaluation (Fedson 2005). Three different groups of healthy rabbits with five rabbits in each (SR1, SR2, and SR3) were maintained. All the containment facilities and biosafety measures were adopted according to instructions provided by Biosafety Level 2 (BSL2) laboratories Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan. In the group, SR1 rabbits were given a single oral dose of 0.5 ml (1×10^{10} CFU/ml) followed by normal feed and fodder. In the group, SR2 an oral dose of 1 ml (1×10^{10} CFU/ml) was given along with normal feed and fodder. Rabbits in SR3 were controls and 0.5 ml of normal saline was orally given to the animals in this group. The body temperature of all groups was measured and dropping samples were collected after every 24 hours till the 7th day of

the experiment. The *Salmonella* viable count was also performed on droppings for evaluation of the recovery of the HB1 live antigen.

Comparative immune response evaluation of live *Salmonella* antigen (HB1) using three different dose levels in rabbits. Four groups of healthy rabbits (10 in each) were maintained in the laboratory animal housing facility attached to the IOM, UAF. Three different dose levels of the HB1 antigen (0.5 ml) were given separately to each group including group R1 with two drops orally (a single dose), group R2 was given two drops orally and repeated after one week (a double dose) and group R3 was given two drops and repeated after alternate days for three times (a triple dose). The group R4 rabbits were kept as inoculated control. All the rabbits were maintained according to the institutional ethics committee recommendation and offered fresh water with feed and fodder *ad-libitum*.

For the detection of antibodies, the titer indirect hemagglutination test (IHAT) of Smith et al. (1977) was used with minor modifications. The live *Salmonella* antigen (HB1) was used to sensitize RBC's using chromium chloride for antigen binding. Two-fold serial dilutions of sera were made in microtitration plate and antiserum raised against HB1 live antigen was placed. The serum raised against HB1 live antigen was diluted separately, and 2% sensitized RBC's were added into 50 µl diluted serum after thorough mixing and were incubated at 37°C for 30 minutes. The different settling of RBC's was interpreted as positive and the compact button formation of RBC's at bottom wells was indicated as negative.

Challenge protection test. Three groups of rabbits R1, R2 and R3 were given an LD50 dose of *S.* Typhimurium (ATCC 14028) (1×10^4 CFU/ml), while the fourth group (R4) was given normal saline and used as a control. The survival percentage for the duration of 70 days and statistical analysis for determination of survival percentage was done using the software GraphPad Prism version 7 (GraphPad Software Inc., USA).

Results

Morphological and biochemical results. The colonies were moderately large (range of 0.5–1.5 mm diameter) convex, glistening and round in shape. In addition, the suspected colonies of *Salmonella* were straw colored with black centers due to the production of hydrogen sulphide (H_2S) on SSA. Typical straw-colored colonies with black centered colonies were further plated for confirmation on BSA. The suspected *Salmonella* colonies were completely black when grown on BSA.

The biochemical characterization of the *Salmonella* isolates was done using a Remel Rapid ID kit (ThermoFisher Scientific, USA) and the results were given in Table III. All ten isolates were confirmed as *S. enterica* (Salmonella I from RapID instruction manual). For quality control, a reference strain of *S.* Typhimurium ATCC 14028 was also tested.

Table III
Results of biochemical characterization by Remel RapID™ and the analysis by ERIC software.

Salmonella isolate	RapID No.	Percentage ID	Spp. identification
TY1	0320410	99.45%	*Salmonella* I
TY2	0320410	99.45%	*Salmonella* I
TY3	0320410	99.45%	*Salmonella* I
HB1	2360410	99.9%	*Salmonella* I
HB11	6363210	99.9%	*Salmonella* I
SP6	6360400	99.9%	*Salmonella* I
CT7	6160400	75.5%	*Salmonella* I
PB2	2300010	98.10%	*Salmonella* I
PB9	2300010	98.10%	*Salmonella* I
HE13	6360400	99.9%	*Salmonella* I
S. Typhimurium ATCC 14028	6360410	99.9%	*Salmonella* I

Molecular characterization of isolates. The DNA extracted from the *Salmonella* isolates was quantified using Nanodrop 8000 spectrophotometer (Thermo Scientific, USA). After DNA quantification the DNA samples were subjected to molecular characterization by PCR and further sequencing of the amplicons. For PCR the 16S rRNA gene was amplified. All ten isolates gave a band in the range of 1.4 kbp after gel electrophoresis shown in Fig. 1. Sequences of all ten isolates were

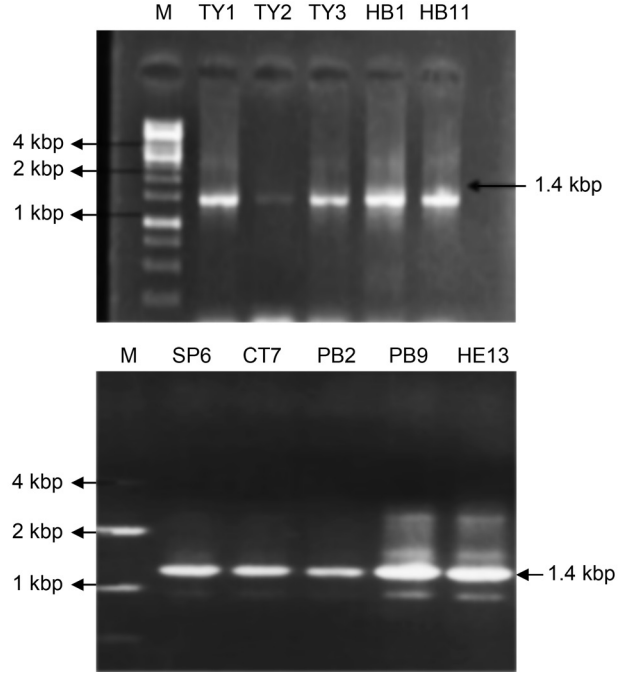

Fig. 1. Amplicons of 1.4 kbp are visible for each *Salmonella* isolate. According to literature, the universal primer (27F, 1492R) gives a PCR product of 1.4 kbp or 1.5 kpb. M is the marker used of 4 kbp size.

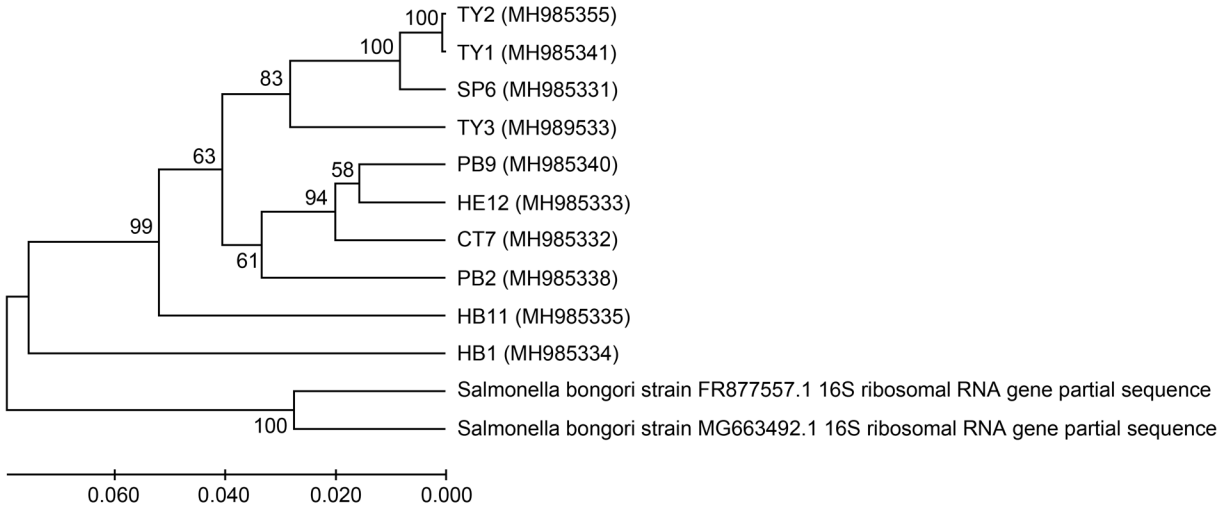

Fig. 2. The phylogenetic tree constructed by MEGA X, with the 16S rRNA sequences of the 10 *Salmonella* strains and two out-groups of *Salmonella bongori*. The UPGMA method was used with 2000 bootstrap replications, bootstrap percentages are indicated on nodes. The ten *Salmonella* species in this study are denoted as previously in this study along with their NCBI accession numbers. Evolutionary distances were computed using the Maximum Composite Likelihood method and they are provided in the units of number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 2). All positions containing gaps and missing data were eliminated. There were a total of 1034 positions in the final dataset.

submitted to NCBI and were allotted following accession numbers; TY1 (MH985341), TY2 (MH985355), TY3 (MH989533), HB1 (MH985334), HB11 (MH985335), SP (MH985331), CT (MH985332), PB2 (MH985338), PB9 (MH985340), HE13 (MH985333). According to the BLAST, the results the three human isolates TY1, TY2 and TY3 had top four hits of *S.* Typhi strains, while HB1, HB11, CT7, HE13, and SP6 had more hits of *S.* Typhimurium with good scores. In case of the poultry isolates PB2 and PB9 both had top hits of *S.* Gallinarum strains.

Phylogenetic analysis of DNA sequences is important in determining evolutionary relationships between species of both prokaryotes and eukaryotes. In this study, an evolutionary tree was constructed by using the Molecular Evolutionary Genetics Analysis (MEGA X) program. The tree construction was based on the *Salmonella* 16s rRNA sequences obtained in this study and two out-groups of *S. bongori*. According to the tree results (Fig. 2), the sequences were denoted as previously along with their NCBI accession. Alignment of the sequences was done using Multiple Sequence Comparison by Log-Expectation (MUSCLE). The statistical method used for inference of evolutionary history was the Unweighted Pair Group Method and Arithmetic Mean (UPGMA).

Looking at the tree in Fig. 2, various clades can be observed. Two of the strains isolated from humans; TY1 and TY2 shared the most common ancestor (gene) forming a monophyletic clade, also the 16S rRNA genes of the third strain (TY3) isolated from humans and that of the SP6 strain isolated from sheep were closer to TY1 and TY2 from an evolutionary standpoint. Another monophyletic clade of the HE 13 strain isolated from horse and the poultry isolate PB9 was present in the tree, the second poultry isolate PB9 and the strain (CT7) isolated from cat seemed more related to this clade. The two strains (HB1 and HB11) isolated from honeybee were also similar to each other with respect to their position in the tree.

***Salmonella* plate agglutination test.** The test was performed to determine the homologous and heterologous antibody titer among individual antigens and their antiserum. The assay was performed in a 96-well microtiter plate. All the cumulative results of antigen and antiserum reactions along with all possible combinations (homologous and heterologous) are presented in Table IV. The HB1 antiserum exhibited significant ($p < 0.0001$) cross-reactivity by giving the highest geometric mean titer among all strains (Fig. 3).

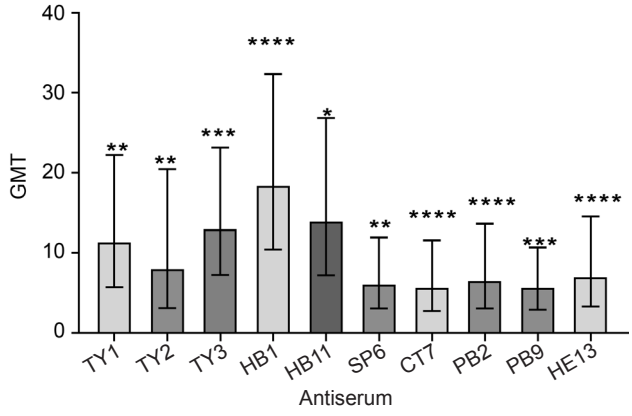

Fig. 3. Geometric mean titers of the antiserum against ten different strains.
Bars indicate the GMT (± SEM) at 95% Confidence Interval (CI) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$).

Table IV
The immunological cross-reactivity assay for the *Salmonella* strains with homologous and heterologous antibody titers and geometric mean titers.

	Antiserum									
Antigen	TY1	TY2	TY3	HB1	HB11	SP6	CT7	PB2	PB9	HE13
TY1	1:64	1:32	1:16	1:16	1:16	1:2	1:4	1:2	1:4	1:8
TY2	1:16	1:64	1:32	1:16	1:32	1:4	1:8	1:4	1:2	1:4
TY3	1:16	1:32	1:64	1:8	1:16	1:4	1:2	1:2	1:4	1:2
HB1	1:32	1:16	1:16	1:128	1:32	1:16	1:8	1:16	1:8	1:16
HB11	1:16	1:8	1:16	1:32	1:64	1:8	1:4	1:4	1:8	1:4
SP6	1:4	1:2	1:4	1:16	1:8	1:32	1:2	1:8	1:4	1:2
CT7	1:4	1:2	1:8	1:8	1:16	1:4	1:64	1:8	1:4	1:8
PB2	1:8	1:4	1:8	1:16	1:8	1:16	1:8	1:64	1:16	1:8
PB9	1:4	1:2	1:8	1:16	1:4	1:2	1:4	1:8	1:32	1:8
HE13	1:8	1:14	1:8	1:16	1:4	1:4	1:4	1:4	1:2	1:64
GMT	11.31	8	13	18.38	13.93	6.063	5.657	6.498	5.657	6.964

Safety evaluation of HB1 live antigen. Zero mortalities were observed among the rabbits of the two groups SR1, SR2 immunized with different doses of HB1 live antigen (1×10^{10} CFU/ml). Overall, in the ten rabbits, no physical abnormalities were observed, while the feed intake remained normal throughout the seven days with no apparent weight loss. Colony count was performed on the feces of the five rabbits throughout the experiment and recovery of the *Salmonella* HB1 live antigen from the five rabbits was shown to be between 1×10^3 to 1×10^5 CFU/ml for SR1, and 1×10^5 to 1×10^8 CFU/ml for SR2. The complete results for the two immunized groups and the controls (SR3) given normal saline are given in Table SI of the Supplementary information.

Immune response in rabbits to different doses of HB1 live antigen. The rapidity of the immune response was similar for the groups R2 and R3 with a GMT of 12.1 for both groups 10 days post vaccination. For the group R2, which were given the double dose, the highest GMT of 119.4 was observed at 40 days post-vaccination; while the lowest GMT of 8 was observed at 70 days post-vaccination. The group R1, given the single dose of HB1 antigen, initially exhibited low immune response with a GMT of 9.1 and 17.1 at 10 and 20 days post vaccination (Table V). However, there was a rise in the immune response of the rabbits with a GMT of 73.5 at 50 days post vaccination. The lowest GMT of 6.6 was recorded at 70 days post-vaccination. The group R3, given tripe dose of the HB1 live antigen, showed a strong immune response at 40 days post vaccination with a GMT of 122.4, and the lowest GMT of 5.8 at 70 days post-vaccination.

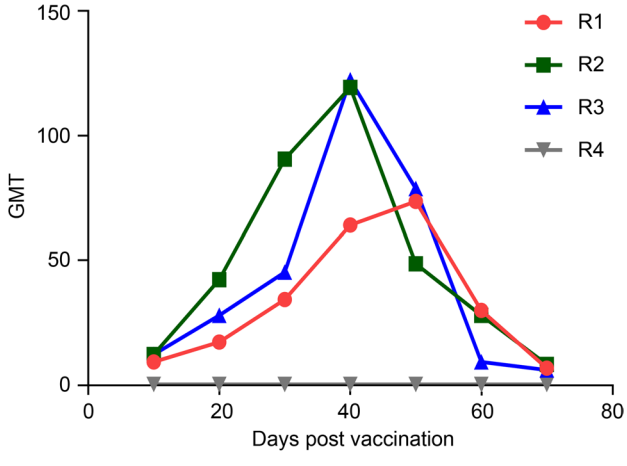

Fig. 4. Comparative indirect hemagglutination titers GMT in the three vaccinated groups and the control group during the 70-days animal trial.

Results of challenge protection test. Protective efficacy of the three different doses of HB1 live antigen in rabbits (R1 = single dose, R2 = double dose, R3 = triple dose, and R4 = control) was evaluated against LD50 dose of *S.* Typhimurium (ATCC 14028) (1×10^4 CFU/ml) for a duration of 70 days post immunization. The LD 50 dose was determined using the method of Reed and Muench (1938). The survival rates of the four groups

Table V
Geomean mean titers of the sera from four group of rabbits immunized with the live HB1 antigen.

Days post vaccination	R1	R2	R3	R4
10	9.1	12.1	12.1	–
20	17.1	42.2	27.8	–
30	34.2	90.5	45.2	–
40	64	119.4	122.4	–
50	73.5	48.5	78.7	–
60	29.8	27.8	9.1	–
70	6.6	8	5.8	–

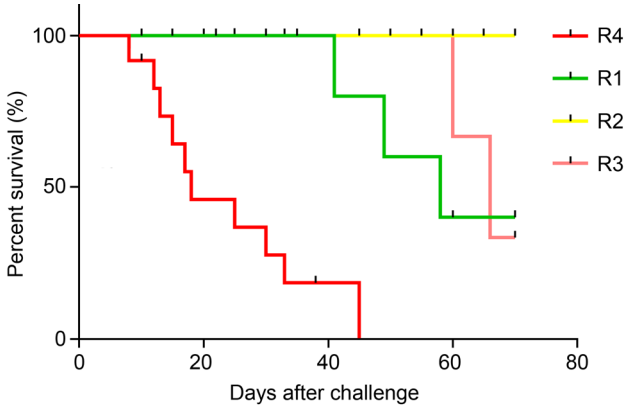

Fig. 5. Survival rate of four groups of rabbits immunized. All four groups were subjected to LD_{50} of *S.* Typhimurium. The three groups (R1, R2, and R3), immunized with different doses of HB1 live antigen, showed a significance increase ($p < 0.0001$) in the survival time in comparison to the control group (R4). Each group consisted of 10 rabbits. Kaplan Meier graph was generated by GraphPad Prism 7.

(each group included ten rabbits) are given in Fig. 5 in the form of a Kaplan Meier graph (it is also given in Table SIII as percentage protection). The three vaccinated groups that received three different doses (HB1 live antigen) had significantly longer survival rates as compared to the control (R4; given normal saline, NS), $^{****}p < 0.0001$. All the rabbits injected with NS succumbed to the *Salmonella* infection by day 45th day of the test. However, the R2 had zero mortalities throughout the 70-day period of the test, while R1 and R3 had three and two mortalities, respectively. The double dose of the HB1 live antigen (0.5 ml) proved to be the most efficient dose in the challenge protection test.

Discussion

Salmonella-related infections are a major disease threat in Southeast Asian countries including Pakistan. With antibiotic resistance in *Salmonella* strains increasing at an alarming rate, the role of antimicrobial therapy for treating infections appears to be ineffective in the battle against multi-drug-resistant (MDR) strains. Moreover, the situation could be altered by effective strategies that include: caution in the excessive use of antimicrobials, better diagnostic techniques and the finding of effective novel antimicrobials. Vaccines have been elected as a suitable measure to prevent the occurrence of *Salmonella* infections. The present research was designed to evaluate the antigenic cross-reactivity of *Salmonella* strains isolated from different human and animal sources in Faisalabad, Punjab, Pakistan. The predetermined goal of the research was to find an avirulent *Salmonella* strain, whose live antigen would prove to be a potential candidate for the development of a live oral cross-reactive vaccine. The vaccine would be trialed in an animal model for evaluation of efficacy.

There was a variation among the prevalence percentage of the *Salmonella* strains isolated from different hosts. The highest prevalence was found in humans with a percentage of 20%, followed by the honey bee (13%), poultry birds (13%), and cat, sheep, and horse, all three having prevalence percentage of 6%. In Pakistan, prevalence studies on *Salmonella* have regularly pointed towards the higher prevalence of *S.* Typhi in human subjects (Ochiai et al. 2008). In another prevalence study in Karachi, Pakistan, the prevalence percentage of *S.* Typhi from human subjects was reported to be 9.1% (Siddiqui et al. 2015). The prevalence percentage of *S.* Typhi in the human subjects in this study was the highest among all the *Salmonella* strains. Orji et al. 2004, have reported a high prevalence of *S.* Gallinarum and *S.* Pullorum in poultry birds in Pakistan with a combined percentage prevalence of around 12% for both strains. In our study, one strain of both Salmonella serovars was found with a combined prevalence percentage of 13%. Interestingly, in our study the occurrence of two *Salmonella* strains from the gut of honeybee was unexpected. The honeybee nests selected were not situated in the vicinity of any sewer, however, the contact of the bees with any animal feces cannot be ruled out. Also, Tarpy et al. (2014), have reported the presence of members of the Enterobacteriaceae family in the gut microbiome of the honeybee, which was one of the main reasons why we chose honeybee in our insect category for the sampling of *Salmonella* spp.

The targeting of the 16S rRNA gene in bacteria for numerous phylogenetic and taxonomic studies is becoming quite common. There are several reasons why the scientific community has resorted to this gene as the housekeeping marker, i) it is ubiquitous in all bacteria, and may exist as a multigene family or an operon, ii) the sequence of the gene is highly conserved, indicating that random sequence changes could be more useful in determining evolutionary pathways iii) the size (1400–1500 bp) of the 16S rRNA gene makes it a suitable candidate for bioinformatics analysis (Janda and Abbot 2007). Based on the sequencing results generated after the amplification of the 16S rRNA given in the Results section, the ten isolates were confirmed to be *Salmonella* strains. Previous results of Trkov and Avgustin (2003), also validated the success of the primers 27F, 1492R used in this study for the amplification of the 16S rRNA gene of different strains of *S. enterica*. The phylogenetic analysis of the sequences yielded interesting evolutionary relationships among the ten strains (sequences submitted to NCBI) of this study.

The scientific community has envisioned a cross-reactive live vaccine for enteric bacteria (Chedid et al. 1968). Various reports (Chacana and Trezolo, 2006;

Matluva et al. 2013) have confirmed the cross-immunity induced by live *Salmonella* vaccines in animal models. However, a live vaccine is only considered effective against an intracellular pathogen such as *Salmonella*, if it can induce both systemic (IgG) and mucosal (IgA) immune responses. In the present research, the double dose (two drops of 0.5 ml and repeated after one week) of the HB1 vaccine gave the best protection in the rabbits in response to LD50 of *S*. Typhimurium, as indicated by the challenge protection test. However, a shortcoming of this study was the determination of the type of humoral immune response (either systemic or mucosal) taking place in the rabbits. As *Salmonella* is an enteric pathogen, IgA is more likely to be the dominant player in the protective immune response after secretion into the gut lumen. As IgG (systemic antibodies) are more likely to combat *Salmonella* bacteria, which have left infected cells and migrated to other tissues, thus initiating new infections (Nandre et al. 2015).

Preliminary results do support the avirulent live HB1 strain to be a highly effective cross-reactive live vaccine in rabbits, which works in a dose-dependent manner. However, due to certain limitations, the exact mechanism of this cross-protection elicited by the vaccine could not be established. It has been recognized that live *Salmonella* strains are detected by the host immune system through the surface exposed antigens, as cell envelope shields the internal antigens (Burton et al. 2014). The mechanism of action of the HB1 live vaccine could be synonymous to the above-mentioned hypothesis. According to literature, most of the identified cross-protective antigens of *Salmonella* are surface exposed (Baumann 2014). On the cell surface of *Salmonella*, lipopolysaccharide (LPS) has been considered the main component to induce cross-reactive antibodies in the host. Various experiments have been performed on the complete core LPS and truncated LPS of *Salmonella* strains along with their cross-protection (Nnalue 1999). However, literature reported previously, supports the argument that the common outer membrane proteins (OMPs) such as OmpD and the core LPS *Salmonella* can induce cross-protection. As the core region of the LPS in *Salmonella* serotypes is highly conserved; this could be a candidate for studies that could help explain the cross-protection induced by the HB1 vaccine. However, the role of cell-mediated immune (CMI) responses cannot be overlooked as live oral *Salmonella* vaccines have been shown to elicit cross-reactivity by stimulating these CMI responses in the host, and future studies on the CMI responses induced by HB1 vaccine would be helpful.

Various avirulent live attenuated *Salmonella* mutants have been used as vaccine candidates in the past. These mutants have certain gene knockouts for attenuation and have been extensively studied. The use of attenuated strains bears various advantages such as the ability to carry heterologous antigens and to induce humoral, cellular and mucosal immune responses. However, the HB1 strain was not attenuated due to the lack of genomic information about the strain and the safety evaluation test gave us the notion to use it as a whole live antigen. For the preservation of the HB1 vaccine honey (2%) was used. However, how this preservative affected the vaccine should be evaluated in future studies. Various preservatives are used in vaccine preservation; some of these include aluminum salts, thimerosal (mercury-containing preservative), human serum albumin, and formaldehyde. Future studies could be performed using various preservatives and checking the stability of the vaccine (a stability trial was conducted whose results are given in the Supplementary file).

This research could prove to be just the tip of a huge iceberg with numerous areas yet to be explored. However, it does lay down the foundation of a *Salmonella* strain that has shown significant cross-reactivity against homologous and heterologous strains and significant efficacy as a vaccine against lethal infection by *S*. Typhimuirum. First of all, a future perspective should be the whole genome sequencing of the strain, as this study just confirmed the HB1 isolate as a *Salmonella* strain by sequencing of the 16S rRNA gene. A whole genome sequence would certainly be helpful in the identification of the suitable conserved antigens in comparison with already sequenced *Salmonella* strains. After comparative genomics of the strains, the development of HB1 strain mutants may be plausible, and these mutants could also be evaluated as future vaccine candidates in comparison to the wild type. In addition, the proteomic analysis including 3-D structural analysis of the structure of the proteins of the HB1 strain could also be helpful in identifying the amino acid differences that might be critical for the cross-reactivity of the antigens of the strain.

The animal model included specific pathogen free (SPF) Chinchilla rabbits infected with LD50 dose of *S*. Typhimuirum (animal pathogen). However, future studies could be orchestrated involving mice infected with other *Salmonella* strains, and to check if the cross-reactive HB1 vaccine still provides significant cross-protection. In this regard, the prospective studies could involve humanized mice (Mian et al. 2011) infected with *S*. Typhi strains. These mice have been used to mimic *S*. Typhi infections and have proven to be a valuable tool in studying the pathogenesis of *S*. Typhi. Future research could apprehend if the HB1 vaccine could provide similar cross-protection against *S*. Typhi strains in humanized mice.

Conclusion

The HB1 live oral antigen (double dose) elicited a strong immune response in the rabbits used in the animal trial, this vaccine could also prove to be a good

candidate as a potential vaccine in animals against other strains of *S*. Typhimurium. Overall, this study does support the notion that the HB1 vaccine can be an effective live oral vaccine that may provide cross-protection against other *Salmonella* strains in an animal model and may also be tested for immunoprotection against *S*. Typhi in a humanized mouse model. However, only rigorous future experiments would be able to corroborate the claims about the immunoprotection potential of the HB1 live oral antigen.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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