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ORIGINAL ARTICLE

Immunological characterization of chitosan adjuvanted outer membrane proteins of *Salmonella enterica* serovar Typhi as multi-epitope typhoid vaccine candidate

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Abstract

Background Outer membrane proteins (OMPs) of Gram-negative bacteria have been known as potential vaccine targets due to their antigenic properties and host specificity. Here, we focused on the exploration of the immunogenic potential and protective efficacy of total OMPs of *Salmonella enterica* serovar Typhi due to their multi epitope properties, adjuvanted with nanoporous chitosan particles (NPCPs).

The study was designed to extrapolate an effective, low cost prophylactic approach for typhoid fever being getting uncontrolled in Pakistan due to emergence of extensively drug resistant (XDR) strains.

Methods & results The OMPs of two *S.* Typhi variants (with and without Vi capsule) alone and with nanoporous chitosan particles as adjuvant were comparatively analyzed for immunogenic potential in mice. Adaptive immunity was evaluated by ELISA and relative quantification of cytokine gene expression (IL4, IL6, IL9, IL17, IL10, TNF, INF and PPIA as house keeping gene) using RT-qPCR. Statistical analysis was done using Welch's test. The protection was recorded by challenging the immunized mice with 50% ×LD50 of *S.* Typhi. The Vi + ve-OMPs of *S.* Typhi showed the most promising results by ELISA and significantly high expression of IL-6, IL-10 and IL-17 and 92.5% protective efficacy with no detectable side effects.

Conclusion We can conclude that the OMPs of Vi + ve *S.* Typhi are the most promising candidates for future typhoid vaccines because of cost effective preparation without expensive purification steps and multi-epitope properties. Chitosan adjuvant may have applications for oral protein based vaccines but found less effective in injectable preparations.

Keywords Outer membrane proteins · chitosan · Adjuvant · ELISA · Relative quantification · Vaccine

Introduction

Salmonella enterica serovar Typhi (*S.* Typhi) is the causative agent of a systemic human infection, typhoid fever, more commonly called enteric fever. High annual global incidence of typhoid fever poses a serious threat to human health. The most recent estimates suggest approximately 11 to 21.7 million cases and untreated / treatment failure cases leading to 128,000 to 2,18,000 typhoid-related deaths annually worldwide [1]. Most of the global typhoid burden is shared by South East Asia where typhoid fever remains endemic and Pakistan has been declared at the most risk of typhoid incidence, due to recent outbreak of extensively drug resistant typhoid in Sindh [2, 3] and also from some parts of Punjab [4]. The incidence data of XDR *S.* Typhi emphasize on the underpinning efforts to expand access to typhoid vaccines. The currently available vaccines are TCV, Ty21a and Vi-CPS with about 81 – 55% efficacy

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respectively [1, 2]. After the emergence of Vi-ve isolates the vaccines that were designed against Vi antigen also need consideration [5]. It was considered that Vi is the main virulence factor of *S.* Typhi and most of the available vaccines are based on Vi antigen. TCV is the latest and WHO prequalified Vi conjugate vaccine which is recently introduced in EPI program of Pakistan, but its efficacy may be affected due to natural occurrence of Vi-ve strains of *S.* Typhi in Punjab [6]. More focused efforts are required to develop vaccines that are more immunogenic for the local strains and can be prepared locally in countries of endemicity [7].

The current study is designed to extrapolate the immunogenic potential of a mixture of total OMPs of *S.* Typhi as independent vaccine candidate. The outer membrane of Gram-negative bacteria is a continuous structure on the outer surface of bacteria, facing directly the host immune system. Its outer component is constituted by lipopolysaccharide (LPS) and proteins. OMPs serve as virulence factors for nutrient scavenging and evasion of host defense mechanisms. Most of the studies have focused on the outer membrane bacterial proteins as immunogens in their recombinant form, capable of eliciting immune response against *Salmonella*, but having less protective efficacy[8].

In this study, total outer membrane proteins of two variants of *S.* Typhi (Vi + ve and Vi-ve strains) are evaluated for their protective role independently and also, with nanoporous chitosan particles (NPCP) as adjuvant to provoke immune response in mice [9, 10]. The rationale was to exploit the multi-epitope properties of multiple proteins for activation the immune response to an adequate level. Adjuvants may stimulate the immune system using a combination of various mechanisms including formation of depot, induction of cytokines and chemokines, recruitment of immune cells, enhancement of antigen uptake and presentation, and promoting antigen transport to draining lymph nodes [11]. In the current study we have evaluated the nanoporous chitosan particles, as adjuvant due to its described properties that is “non-toxic, biocompatible and bio-degradable” and large surface area due to nanoporous nature. Because of these valuable properties, chitosan offers advantages for adjuvant and vaccine delivery systems [12, 13].

We isolated and purified the *S.* Typhi OMPs and prepared their formulation with nanoporous chitosan particles (NPCPs-OMP). Both OMPs and NPCP-OMPs preparations for Vi + ve and Vi-veisolates were evaluated for the humoral and cell mediated immune response. The protective role of both the approaches was also compared against *S.* Typhi in mice as model animal.

Materials and methods

Culture and identification of Vi + ve and Vi-ve *S.* Typhi isolates

Salmonella enterica serovar Typhi (*S.* Typhi) isolates, two Vi + ve (NIB 40 & NIB 21) and two Vi-ve (NIB 27 & NIB 63), were taken from already preserved glycerol stocks (-20°C) from Enteric Pathogens Lab, National Institute for Biotechnology and Genetic Engineering (NIBGE, Pakistan). These isolates were revived in tryptic soy broth (TSB, Merck, Germany) and streaked on MacConkey Agar plates (65 g/ 1000 mL [w/v]) for isolation and purification. A well isolated and clear colony from each MacConkey agar plate was picked and stabbed on triple sugar iron (TSI) agar slant for biochemical identification.

The DNA of biochemically identified isolates was extracted by phenol-chloroform method and subjected to PCR targeting *S.* Typhi specific *fliC* gene [14, 15]. The confirmed *S.* Typhi isolates were screened for presence of Vi gene by PCR targeting *tviB* gene [16]. The PCR conditions were same for both genes. A 25 µl DNA amplification mixture contained 2·5 µl of 10x Taq Buffer, 1·5 µl of 25 mM $MgCl_2$, 1 µl of 10 pmol of each primer, 0·75 µl of 2·5 mM dNTP, 0·2 µl of Taq polymerase, 5 µl of template and distilled water to make up the volume. The reaction mixture was subjected to 30 cycles of 1 min each at 94 °C (denaturation), 50 and 52 °C (annealing) for *fliC* and *Vi* gene respectively, and 72 °C (extension) followed by incubating at 72 °C for 5 min. The primer sequences and annealing temperatures are given in supplementary **Table S1**.

Isolation and purification of outer membrane proteins of *S.* Typhi

The OMPs of the confirmed Vi + ve and Vi-ve *S.* Typhi isolates were extracted according to the protocol described by Hamid and Jain et al. with minor modification [17].

Briefly, one liter culture of both Vi + ve and Vi-ve strains in TSB was incubated overnight at 37 °C in a shaking incubator at 100 RPM. Cells were harvested by centrifugation at 6000 RPM for 30 min at 25 °C. The cell pellet was washed with 10 mM Tris-HCl buffer and weighed. OMPs of one gram of bacterial pellet was extracted for 1 h at 4 °C with vigorous stirring by using 20 ml of extraction buffer [Extraction Buffer: 6 M urea; 10 mM EDTA; 10 mM T buffer]. The OMP extract was dialyzed (renaturation) against distilled water for 2 days to remove urea, with changing water at regular intervals of eight hours. The dialyzed extract was centrifuged at 6000 RPM for 1 h at 4°C. The clear supernatant containing OMPs was aliquoted in falcon tubes (50 ml) and stored at -20 °C for freezing the extract, followed by lyophilization.

Analysis of OMPs on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The concentration of the OMPs was estimated by nano-drop (2000c Thermo Scientific). The purity and integrity of extracted OMPs was checked on SDS-PAGE according to method developed by Ulrich K. Laemmli [18]. Briefly, the OMP samples 20 µg / 20 µL were solubilized in sample buffer (0.625 M Tris-HCl; pH-6.8, 2% sodium dodecyl sulphate (SDS), 10% glycerol and 5% β- mercapethanol) and heated at 100°C for 5 min. Samples were carefully loaded into the wells along with 5 µL Novex pre-stained protein standard. The initial running voltage was set to 60 V until the sample reached the interface of resolving gel (discontinues buffer system). At this point, the voltage was changed to 110 V and electrophoresis was continued till the tracking dye reached the bottom of the gel. The gel was stained with staining solution [Acetic acid 10%; Iso-propanol 25%; Coommassie Blue R-250 (0.025%)] for 1 h followed by de-staining with 10% acetic acid overnight. The gel was visualized on white light and the OMPs were analysed by comparing their sizes with standard.

Synthesis of nano-porous chitosan particles (NPCPs)

Chitosan from crustacean's chitin (MP Biomedicals, France), acetic acid 99.7% (Acros organics, USA), tween 60 (Acros organics, USA), linoleic acid (Acros organics, USA), sodium tripolyphosphate (Bio-Basic Inc.) were used as starting material without further purification.

Nano-porous chitosan particles (NPCPs) was prepared by ionic gelation method with few modifications of previously described method [19]. In detail, 0.6% of chitosan was weighed, mixed into 1% acetic acid solution and left overnight with continuous stirring at 25 °C temperature. Five milli liter of the prepared solution was heated at 55-60 °C for five minutes, followed by addition of 75 mg of Tween-60 with continuous stirring for 30 min. One drop of linoleic acid was added dropwise into the solution and stirred for 20 min. Five milli liter of 0.5% tri-polyphosphate was added dropwise and the solution was stirred for 30 min at 50 rpm. After cooling to 25 °C temperature, it was centrifuged at 7000 rpm for 10 min. The pellet was washed with ethanol and water three times. The obtained pellet was lyophilized (freeze dried) to make the powder.

Attachment and characterization of OMPs with NPCPs

The prepared NPCPs were weighed and dispersed in phosphate buffer saline (PBS) having pH 7.4 (1 mg NPCPs / 1ml PBS). Similarly, both proteins (Vi + ve OMPs & Vi-ve OMPs) were dissolved in PBS [1 mg protein /1 ml PBS (1X)]. Both NPCPs and OMPs were mixed in 1:1 ratio and incubated for 1 h at 25 °C on a shaker at 100 rpm. Samples were analyzed by Malvern Zeta sizer Nano ZS with DTS Nano software. The purpose of this preparation was to check whether the OMPs and NPCPs were compatible to each other and whether they had bio-absorbable charge or not.

Characterization of NPCPs- OMP complexes

DLS studies

The size and zeta potential of the prepared NPCPs alone and those conjugated with OMPs were analyzed by Malvern Zetasizer Nano ZS with DTS Nano software.

Scanning electron microscopy

Field emission scanning electron microscope (FESEM, JSM 7500 F; JEOL, Tokyo, Japan) at accelerating voltage (1–30 kV) was used to study the morphology of the NPCPs.

Fourier transmission Infra-Red (FTIR) spectroscopy

FTIR (Bruker Alpha Platinum ATR) was used to analyze the sample. Twenty milli gram of the prepared sample was taken and spread onto the grid of FTIR. The arm of FTIR was adjusted onto the grid and scanned from 500 to 4000 cm^{-1}. The spectra were drawn and analyzed with Origin pro software.

Evaluation of outer membrane proteins with and without NPCP-adjuvant for immunogenic potential

To evaluate the immunogenic response of OMPs of *S.* Typhi with and without NPCPs, the indicators selected were humoral immune response by indirect ELISA, cell mediated immune response by expressional analysis of cytokine genes and delayed type hypersensitivity, taking mice as a model organism.

For preparation of OMPs conjugated with NPCPs that can be delivered to the model organism, 0.2% lyophilized nano-particles were dissolved in 25 mM Sodium Acetate (pH 4.8) followed by addition of 2 µg / µL OMPs of *S.* Typhi. The preparation was kept at room temperature for

16 h to achieve maximum binding of NPCPs with OMPs. Similarly, 2 µg/ µL OMPs alone were dissolved in 25 mM Sodium acetate (pH 4.8) and the concentration of OMPs was calculated by nanodrop method. Four formulations were prepared in the experiments for evaluation of immunogenic potential: Vi + ve OMPs, NPCP-Vi + ve, Vi-ve OMPs, NPCP-Vi-ve.

Evaluation of humoral immune response by indirect ELISA

Indirect ELISA was used to study the humoral immune response of our candidate antigens in the mice as the experimental animal. This study was performed in line with SOPs of The Institutional Animal House Committee at National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan which granted approval (No.193; dated 26-07-2019).

For this purpose, antisera were raised in animals as follows: five groups of mice were included in the study, each group consisting of ten mice. Four groups were given three shots of the four candidate antigens on alternate days, for three weeks @ 200 µg / 100µL /mouse. Group five was kept as negative control and did not receive any shot [17].

The mice were bleed by cardiac puncture and serum was collected by incubating the blood at 37 ºC for 3 h followed by centrifugation at 4500 RPM for 10 min. The collected sera were used for indirect ELISA testing. ELISA was performed according to the procedure described by Christopouloset al.[20]. Briefly, ELISA plates were coated with OMPs (from both Vi-ve and Vi + ve strains) as antigens prepared at a concentration of 4 µg/ 200 µl of PBS having pH 7.4, and incubated overnight at 4 °C. Each plate was washed thrice with PBS and blocked with 5% BSA to avoid non-specific binding. The serial dilutions (1:1, 1:200, 1:400, 1:1600, 1:3200 and 1:6400) of sera collected from immunized mice were added in the respective wells as primary antibodies and incubated for 1 h at 37 °C. The ELISA plate was washed thrice with PBS and secondary antibodies i.e. anti-mouse IgG antibodies conjugated with horseradish peroxidase (HRP) were added (1:20,000 dilution) and incubation was done for 1 h at 37°C. After incubation, the ELISA plate was washed thrice with PBS and the HRP substrate, TMB was added for colour development [21]. Microtiter plate reader equipped with a 550 nm filter (for HRP) was employed for reading. Graph was designed using comparative analysis of control with test materials, the confidence interval was kept 95% Cl all the results were statistically analyzed by ANOVA test.

In vitro evaluation of cell mediated immune response by relative quantification of cytokine gene expression

The cell mediated immune response to candidate antigen was evaluated by estimating the cytokine gene expression. The relative quantification of expression of cytokine genes was performed by real time RT-PCR. Five groups of six female BALB/c mice, aged 6–8 weeks old were immunized by the intraperitoneal (I.P.) injections at a dose of 0.1 ml per mouse as described in Sect. 2.7 and 2.8. The blood was collected by cardiac puncture after 24–36 h in EDTA vacutainer for further analysis. Soon after collecting blood, RNA was extracted by Trizol reagent according to the instruction provided by the manufacturer (Trizol, Invitorgen). The RNA is highly unstable so it was immediately proceeded for the synthesis of cDNA using random hexamer primers. All the cDNA samples were quantified on nanodrop to equilibrate the value of all the samples followed by real time PCR for relative quantification of cytokines gene expression.

For this purpose, 8 genes i.e., IL4, IL6, IL9, IL17, IL10, TNF, INF and PPIA (housekeeping) were studied. The primer sequences are given in Table S1. All the reactions were run in triplicates along with internal control and non-stressed samples. For the real time analysis, Syber green master mix (Thermofisher Scientific) was used according to the instructions provided by manufacturer. The real time expression was measured by using equation R = 2 (ΔCt sample – ΔCt control) or R = 2 (-ΔΔCt) [22]. The ratio of gene expression was calculated by using formula:

$$ratio = \frac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}}$$

Statistical analysis Cell mediated immune response involves the activation of phagocytes, and antigen-specific cytotoxic T-lymphocytes indicated by expression of several cytokines. The data sheet obtained from qRT-PCR machine was further analyze by using equation R = 2 (ΔCt sample – ΔCt control) or R = 2 (-ΔΔCt). The conclusive graphical interpretation of all genes presented the comparative expression of cytokines and T-lymphocytes with the normal control (normal level of analytes). The data were normalized using PPIA gene as the reference housekeeping gene. Statistical analysis was done using Welch's t test in GRAPHPAD Prism version 8.4.3. A P value < 0.05 was considered statistically significant. The CI (confidence interval 95%) and relative gene expression was also calculated to validate the results.

Protection studies

Protection studies were performed on five groups of female mice, aged 6–8 weeks, each having ten animals. The mice were immunized by the intraperitoneal injections (0.1 ml per mouse) as described in Sect. 2.7 and 2.8. The mice got five shots of injection on day 0, 7, 21, 28 and 40 and were then challenged with bacterial dose of 50×LD50 (5×10^6 CFU/mL) calculated via serial dilution and plating method, by injecting ~0.7 OD at 695 nm directly into intraperitoneal cavity [23]. The mice were strictly observed for 24 to 36 h for development of morbidity or mortality. Routine observations were continued for 14 days of post injection to get the mortality count.

In vivo cell mediated immune response by delayed type hypersensitivity response

The footpad swelling method described by Collins and Mackaness (3) was used to carry out the delayed-type hypersensitivity (DTH) studies. Delayed-type hypersensitivity is an inflammatory response induced mainly by T cells when a substance is recognized as antigen by the immune system. The animals were divided into five groups of four animals each, with one group serving as the control. The two test groups received a single injection each, of 25 µg/ 10 µL of OMPs dissolved in PBS (conditions were same for both Vi + ve and Vi-ve OMPs) in their right hind footpads, while the left footpads received an injection of an equal volume of PBS serving as the control. The other two groups received 25 µg/ 10 µL of NPCPs-OMPs (for both Vi + ve and Vi-ve OMPs) in their right hind footpads, left footpads got a shot of 10 µL of PBS. The control group of animals was given the injection of an equal volume of PBS instead of OMP preparations into their right and left hind footpads. The footpad swelling was measured at different time intervals (3, 6, 24, 48, and 72 h of post injection) with the help of a digital Vernier caliper.

Results

Confirmation of encapsulated (vi + ve) and non-capsulated (Vi-ve) *Salmonella enterica* serovar Typhi isolates

The bacterial cultures were identified on the basis of colony color and morphology, i.e., translucent, round and small, 2–3 mm colonies were selected as *S.* Typhi positive. The typical reaction on TSI slants was observed as alkaline (red/pink) slant, acidic yellow butt and slight H_2S production indicated with black color interface. The purified typical bacterial culture was identified by PCR for *fliC* gene which produces a 363 bp amplicon in case of positive culture. The Vi antigen variants were identified by *Vi* gene-based PCR which gives a 599 bp amplicon in Vi positive strains only.

Electrophoretic profile of outer membrane proteins of *S.* Typhi

A complex electrophoretic profile of proteins was found in the outer membrane of Gram- negative bacteria, having more than 16 highly expressing proteins with molecular masses between 10 and 110 kDa (**Supplementary Figures and Tables, Fig. S1**). The molecular masses of 16 main proteins were calculated to be 110, 78, 63, 55, 49, 46, 42, 38, 36, 33, 29, 25, 24, 22, 20, 18, and 16 kDa. The yield of OMPs was approximately 4 mg protein/g bacteria (wet weight).

Characterization of nano-porous chitosan particles (NPCPs)

The prepared nano-porous chitosan particles size was confirmed by the zeta sizer through dynamic light scattering analysis. The results suggested that the particle size was almost 955 nm having intensity of 35% which can be seen in the Fig. 1**A.**

While the size of nano-porous chitosan particles conjugated with Vi + ve OMP was 955 nm and Vi-vew as 825 nm

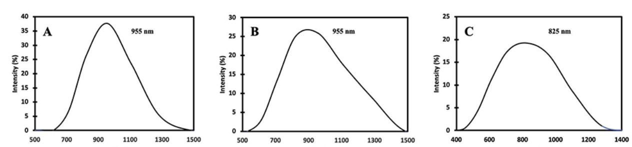

Fig. 1 Size analysis of nano-porous chitosan particles by dynamic light scattering (DLS). ((A) Nano-porous chitosan particles, (B) Nano-porous chitosan particles conjugated with positive potential protein, (C) Nano-porous chitosan particles conjugated with negative potential protein.)

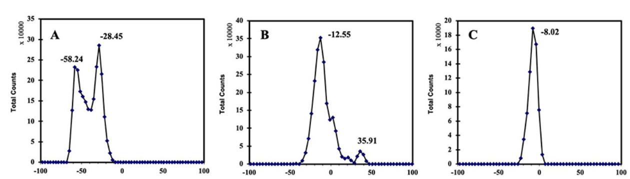

Fig. 2 Overall charge/potential study through dynamic light scattering (DLS). ((A) nano-porous chitosan particles, (B) Nano-porous chitosan particles conjugated with positive potential protein, (C) Nano-porous chitosan particles conjugated with negative potential protein)

as shown in Fig. 1**B**, Fig. 1**C** having the intensity 25% and 18% respectively.

Conjugation of nano-porous chitosan particles with outer membrane proteins

The conjugation of nano-porous chitosan particles with the OMPs were confirmed by zeta potential study through dynamic light scattering analysis. The results showed that nano-porous chitosan particle had overall negative charge peaks (-58.24 and −28.45) which can be seen in Fig. 2**A.** The potential peaks of nano-porous chitosan particle were shifted to -12.55, 35.91 and −8.02 respectively after the conjugation with Vi+ve and Vi-ve OMPs.

The size of nano-porous chitosan particles was confirmed by the scanning electron microscopy. It was seen that overall particle size was ca. 955 nm which is similar to the zeta sizer results. However, one individual particle pore size was almost less than 100 nm in range. This can be seen in the Fig. 3.

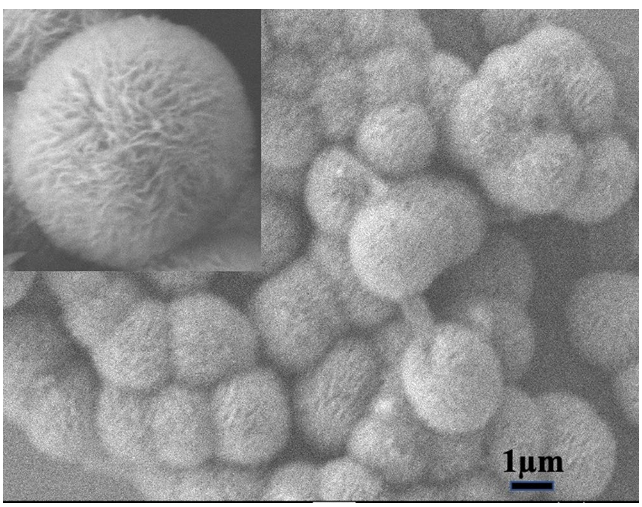

Fig. 3 Scanning electron microscopic analysis of nano-porous chitosan particles.

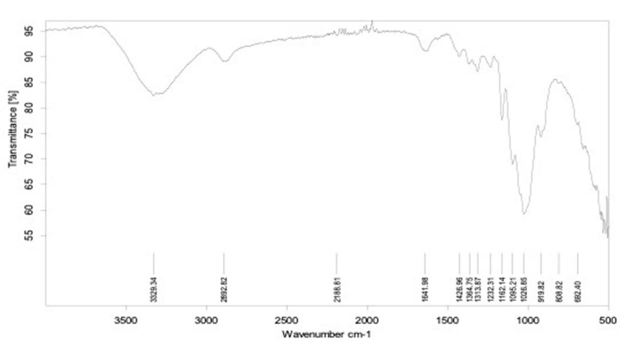

Fig. 4 Fourier Infrared spectroscopic analysis of nano-porous chitosan particles

Furthermore, Fourier infrared spectroscopy analysis was performed to check the presence of functional groups on the surface of nano-porous chitosan particles as shown in Fig. 4. The absorption spectrum between 800 and 1200 cm^{-1} represents characteristics peaks of chitosan saccharides structures, especially the peak at 1026 cm^{-1}represents the C-O-C bonding. While the peaks 2892 and 3329 cm^{-1}represent the C-H and N-H stretching vibrations respectively. The similar peaks can be observed in various groups working on chitosan and derivatives[24–28].

Induction of humoral immune response

The antibody titer in response to Vi+positive OMPs was higher as compared to other preparations, when analyzed by indirect ELISA. The maximum antibody response against

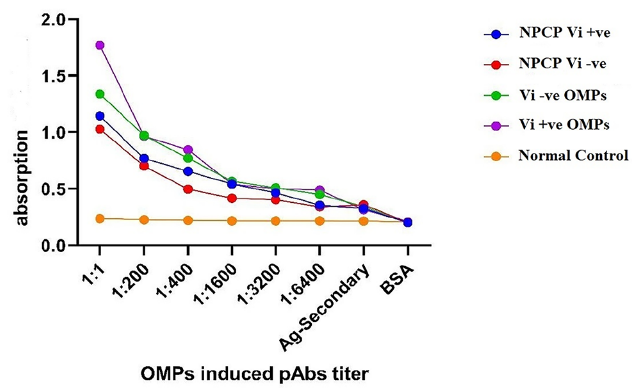

Fig. 5 Induction of humoral response depicted by graphical presentation ELISA results. (The graph depicts the quantitative expression of antibodies raised in mice immunized with OMPs alone and CNP-OMPs by applying ANOVA statistical analysis test: On Y axis the absorbance at 550 nm were taken and on x axis the serial dilution of antigens (OMPs) based antisera in the mice (end point titer), wells that contains only Ag no antibodies and wells having BSA as positive control were taken)

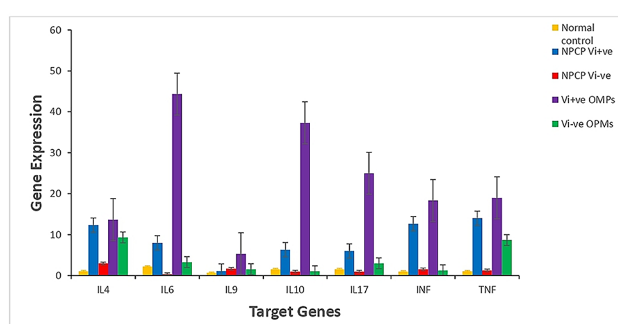

Fig. 6 The graphical presentation of cytokines gene expression by Real-time PCR in response to four test antigenic preparations. (On Y axis the fold change in gene expression is taken and on X axis, the cytokine genes studied during this study are taken)

Vi + ve OMPs was observed as evident by the absorbance value of apx 1.8 at 450 nm. The antibody titre against Vi-ve OMPs, NPCP-Vi + ve and NPCP-Vi-ve were observed in a decreasing sequence showing the highest immunogenic potential of Vi + ve OMPs followed by Vi-ve OMPs, NPCP-Vi + ve and NPCP-Vi-ve respectively. The untreated mice serum and the ELISA reaction with secondary enzyme conjugated antibodies only, were taken as negative control. The humoral immune response against NPCP-Vi + ve OMPs also comparable with Vi + ve OMPs alone. The endpoint titer based ELISA test was performed to check the end point titer of pAbs showing positive results compare to negative control. The results were found statistically significant applying ANOVA test. The graphical interpretation in given in Fig. 5.

Cell mediated immune response

Cell mediated immune response involves the activation of phagocytes, several cytokines and antigen-specific cytotoxic T-lymphocytes. The data sheet obtained from qRT-PCR machine was further analyze by using equation R = 2 (ΔCt sample – ΔCt control) or R = 2 (-ΔΔCt). The maximum increase in the expression level of IL6 showed 44.3 folds increase in the blood of mice injected with Vi + ve OMPs as compared to NPCP-Vi + ve, NPCP Vi–ve and Vi-ve OMPs (8.0, 0.3 and 3.3 folds respectively). The expression level of IL10, IL17, TNF, and IFN were also increased 37, 25, 19 and 18 folds respectively. The level of IL4 (gene controlling the production of T cell) showed 13.8 folds increase in the blood of mice injected with Vi + ve OMPs as compared to NPCP-Vi + ve, NPCP Vi–ve and Vi-ve OMPs (11.9, 2.5 and 8.8 folds) as elaborated in Table 1. The conclusive graphical interpretation of data from all the genes illustrates the comparative expression of cytokines genes with the normal control (normal level of analytes) Fig. 6.

Protection studies

All the animals that were injected with NPCPs-Vi-ve, Vi–ve OMPs and NPCPS-Vi + ve showed the symptoms of salmonellosis, such as ruffled hair, lethargic movements, slow responsiveness to external stimuli at the end of the day 1 post-infection and died after 2 days. 32.5% protection was seen in NPCPs-Vi-ve injected animals. The Vi + ve

Table 1 Immune responsive genes showing significant relative gene expression (fold change) in PBMCs of antigen treated and non antigen treated control mice. The red highlighted values depict the down regulated genes the most up regulated genes were highlighted as bold purple. All the targeted genes showing significant P value along with 95% Ci in the PBMCs of antigen treated mice and non antigen treated normal control mice.

Target gene		Normal control	NPCP + ve OMPs	NPCP-ve OMPs	Vi + ve OMPs	Vi-ve OMPs
IL4	**Fold change compare to Normal control**	1.077	12.33	2.967	**13.67**	9.333
	95% CI value		-20.53 to -1.985	-3.844 to 0.06372	-15.02 to -10.16	-10.68 to -5.831
	P value		0.0112	0.0285	< 0.0001	< 0.0001
IL6	**Fold change compare to Normal control**	2.183	8.000	0.3333	**44.33**	3.300
	95% CI value		-8.994 to -2.639	0.9657 to 2.734	-66.47 to -17.83	-2.066 to -0.1668
	P value		0.0018	0.0006	0.0023	0.0118
IL9	**Fold change compare to Normal control**	0.6733	1.117	1.667	**5.333**	1.567
	95% CI value		-0.8504 to -0.03624	-1.868 to -0.1191	-7.113 to -2.207	-1.632 to -0.1546
	P value		0.0170	0.0132	0.0015	0.0089
IL10	**Fold change compare to Normal control**	1.577	6.333	0.9667	**37.33**	1.050
	95% CI value		-6.485 to -3.028	0.1686 to 1.051	-43.89 to -27.63	0.1191 to 0.9342
	P value		0.0001	0.0064	< 0.0001	0.0074
IL17	**Fold change compare to Normal control**	1.580	6.000	0.9667	**25.00**	3.000
	95% CI value		-6.033 to -2.807	0.3112 to 0.9155	-31.80 to -15.04	-3.033 to 0.1930
	P value		0.0003	0.0007	0.0002	0.0411
INF	**Fold change compare to Normal control**	0.9867	12.67	1.533	**18.33**	1.300
	95% CI value		-20.22 to -5.717	-10.94 to -4.335	-19.57 to -16.37	-10.94 to -4.335
	P value		0.0020	0.0006	< 0.0001	0.0006
TNF	**Fold change compare to Normal control**	1.030	14.00	1.267	**19.00**	8.667
	95% CI value		-17.68 to -5.679	-0.9063 to -0.1870	-31.71 to -2.988	-0.6709 to 0.04422
	P value		0.0013	0.0033	0.0114	0.0459

OMPs gave 92.5% protection against LD50 dose. However, Vi negative OMPs gave only 73.5% protection. Marginal protection was seen in NPCPs-Vi + ve (62.5%), as shown in Supplementary figure, **Fig S2**. The data was analysed by % probability of death rate. The protection studies results were further consolidated by ELISA and real time qPCR base analysis.

In vivo evaluation of-cell immune response

The purified OMPs of both Vi + ve and Vi-ve strains of *S.* Typhi and NPCPs conjugated OMPs were injected to check the swelling pattern in mice footpad. The mice footpad swelling was measured with the help of Vernier caliper at different intervals of time 0, 3, 6, 15, 24, 36, 72 h post-injection. It was found that maximum response induced between 3 and 24 h post injections. DTH response was recorded and it was observed that Vi + ve OMPs and NPCPs-Vi + ve OMPs showed maximum swelling which also persisted for 48 h in case of Vi + ve OMPs, as shown in Fig. 7.

Discussion

The *S.* Typhi infection has become more threatening due to emergence of extensively drug resistant (XDR) strains in Southeast Asia particularly the subcontinent [36]. Being humans as the only reservoir of this pathogen, it is rational to control it by effective immunization. Already available typhoid vaccines have limited efficacy due to the geographical variations in the antigenic components of *S.* Typhi that is the emergence of Vi-ve strains. Latest available Vi conjugate vaccine (TCV) claims for 81% efficacy but it is based on Vi capsular polysaccharide and cannot be protective against the naturally occurring Vi–ve variants of *S.* Typhi [6]. Thus, a better understanding of protective efficacy of antigenic components of *S.* Typhi is needed to consider for more effective vaccines against typhoidal salmonellosis. Previous studies have reported the evaluation of immune response of different *S.* Typhi antigens including LPS, flagellar antigen and O-SP (oligopolysaccharide)[17]. In this study, the OMPs of two naturally occurring variants of *S.* Typhi were compared as total proteins mixture and with

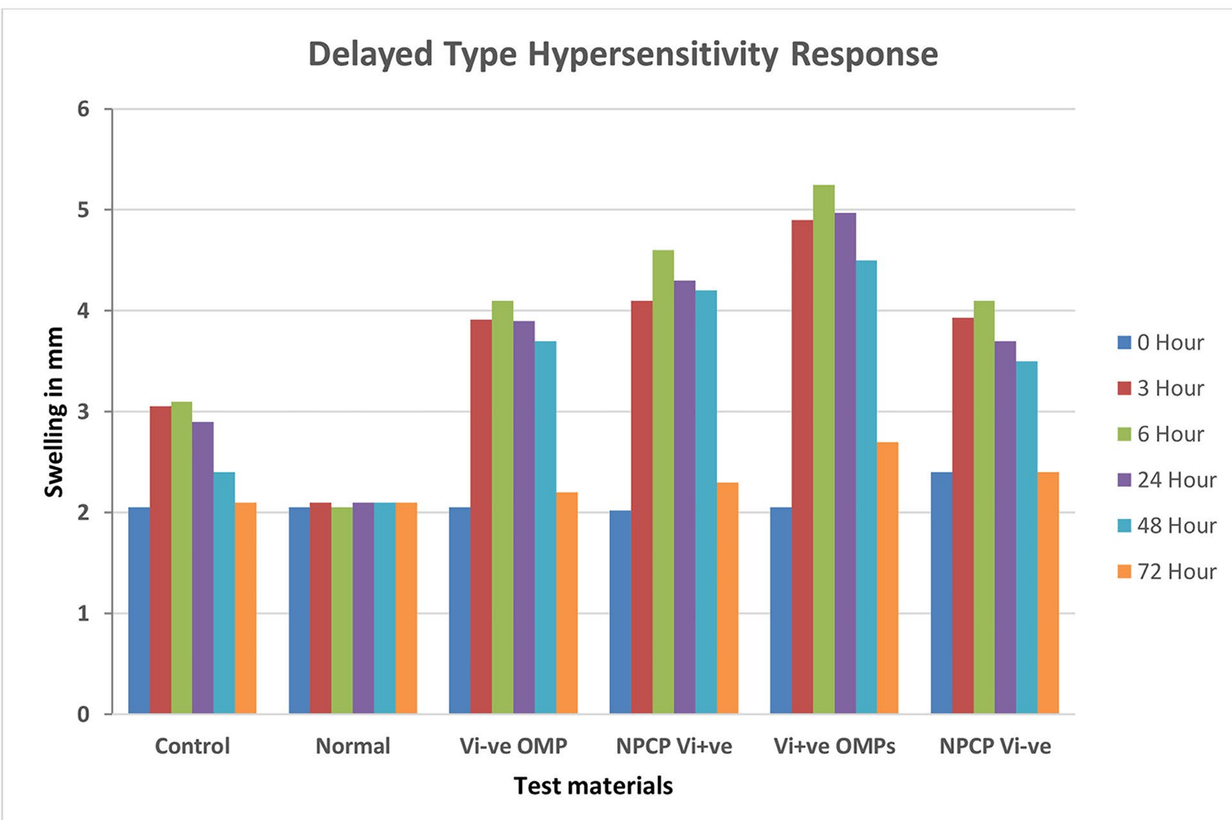

Fig. 7 Delayed type hypersensitivity response to the outer membrane protein preparations. (The graph depicts the physical observed swelling of mice footpad after being injected with OMPs alone and CNP-OMPs. The swelling due to T cells or Natural killer was measured with Veriner caliper: On Y axis the swelling in mm was taken and on x axis hours of observation was taken)

nanoporous chitosan as adjuvant, for their immunological response both *in vitro* and *in vivo* conditions. The OMPs of Gram-negative bacteria have been extensively studied but the information is limited with respect to immunological importance of *S*. Typhi. There is no approved vaccine based on OMPs of *S*. Typhi. After the emergence of XDR strains with incidence 3228/100,000 population it is important to study the significance of OMPs as immunogenic potential vaccine candidates [29, 30]. In this study, we have evaluated adaptive immunity with the help of ELISA for antibody detection and RT-qPCR for relative quantification of cytokines genes expression. Because of the fact that the humoral immune response apart from cellular immune response, is also very important to give protective immunity against pathogen [31, 32]. Our results showed promising humoral as well as cell mediated immune response, induced by total Vi + ve and Vi-ve OMPs as indicated by ELISA with a slight decline in humoral response observed in case of NPCP-OMP preparations. Our findings for crude OMPs were consistent with those of [17], who reported the immunological analysis and protection by a recombinant 49 KDa OMP of S. Typhimurium but the upscaling and hyper production of bacterial membrane proteins for commercial application is tedious and expensive process. Our approach based on the multiple epitopes based vaccine is the most suitable stimulator for generating strong immune response with a low cost and technically simple method. However; our results for OMP-chitosan preparations are contradicting the existing information that these particles show stabilized release of drug and shows substantial improvement in humoral response elicited in response to protein-chitosan (NPCP-OMP) adjuvant preparations [33, 34]. This may be due to diversity in the routes of administration, interaction of target antigen with chitosan, size and ratio of NPCP etc. also discussed by [34], because in our study, we hypothesized that the chitosan might had masked some antibody binding sites on outer membrane proteins or might be denaturing the protein structures resulting in decrease in humoral response.

All the designed experiments were performed on mice model animals, because although exclusively human pathogen, studies revealed that *S*. Typhi when inoculated in peritoneal cavity cause infection in mice [35]. Only limited studies have been taken so far to study the cellular immune response for*S*. Typhi [36]. Cell mediated immune response involves the activation of several immunological pathways through the release of cytokines including IL4, IL6, TNF and INF ¥, which have been reported as important markers. We have assessed the activation of cellular immune response by relative quantification of gene expression of cytokines (IL4, IL6, IL9, IL17, IL10, TNF and INF) along with internal control (PPIA) and external negative control not stimulated with any antigen. The experiments were revised in triplicates for the reproducibility and results were compiled by taking the mean values. The Vi + ve OMPs results in much high expression of IL4 in mice (13.67 fold as compare to normal control 1.077 fold) as compare to Vi-ve-OMPs and NPCPs-Vi + ve, NPCPs-Vi-ve indicating the higher immunogenic potential of Vi + ve-OMPs. The IL4 is used to differentiate naïve helper T cells to Th2 cells after exposure to a potentially immunogenic antigen ultimately activating humoral immunity. The IL6 acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine and releases upon activation of cell mediated immunity. A high expression of IL6 was observed in mice (44.33 fold as compare to NC 2.183 fold) injected with Vi + ve-OMPs. In NPCPs-Vi + ve infected mice, marginally high expression was also noticed.The gene expression of IL10, IL17, INF-γ and TNF increased significantly in response to Vi + ve OMPs followed by NPCP-Vi + ve OMPs. IL10 also functions as anti-inflammatory cytokines; IL17 functions as pro inflammatory cytokines and produced by T helper cells. IL9 regulates the production of a variety of hematopoietic cells. IL9 activates STAT pathway and also stimulates cell proliferation and prevent apoptosis. Interferon gamma is an important activator of macrophages and inducer of Class II major histocompatibility complex (MHC) molecule expression. It is critical for innate and adaptive immunity against pathogens. TNF is activated by macrophages and also associated with CD4 + cells. For all these cytokines and interleukins, it was found that expression increased several folds in Vi + ve-OMPs as compare to all the three others antigens. The expression TNF and IL4 was also found to be increased in NPCPs-Vi + ve, Vi + ve OMPs and Vi-ve OMPs whereas a very low fold change found in NPCPs-Vi-ve mice (Results enlisted in Table 1). The expression of interferon is high in Vi + ve OMPs and NPCP-Vi + ve inoculated mice. The expression of IL17, IL10 and IL9 is much high in Vi + ve OMPs mice as compared to all others. Somewhat similar trend was found with the production of antibodies due to these antigens (Vi + ve OMPs, Vi-ve OMPs, NPCPs-Vi + ve, NPCPs-Vi-ve). Antibody titer is high in case of Vi + ve OMPs, trend decrease from NPCPs-Vi + ve to Vi-ve to NPCPS-Vi-ve. The DTH immune response was also evaluated in this study that is based on the activation of T cells and pro inflammatory cytokines. Maximum swelling was recorded 3 h of post injection. Swelling continues till 36 h after that the trend line of swelling start decreasing. Swelling finally disappears after 72 h. The protection studies result also indicates that Vi + ve OMPS confers more protection to the test animals as compare to Vi negative OMPs which consolidates our finding with cellular immunity and humoral immune response done using sandwich ELISA. All the experiments done in this study have been repeated trice and the conclusive graphs were generated by repeated mean values on which different suitable statistical tests were applied i.e., ANOVA, welch's t test and median lethal dose tests based on %probability of death. It is worth mentioning here that in immunization studies, the challenge was given by Vi+ve isolate which strengthens the hypothesis of limited protection by Vi+ve OMPs against mice immunized with Vi-ve OMPs due to variation in antigenic components. Our study indicates the improved effectiveness and protection of total protein extract as vaccine candidate which is also supported by the findings of [37], which showed the activation of humoral immune response by one recombinant OMP but no protection was observed and author has suggested to use more than one OMP for protective efficacy. Furthermore, as shown in several studies that the mucoadhesive chitosan particles increase the immune response but, in our studies, the flexible nature of chitosan decreases the efficacy of S. Typhi OMPs of. That might be due to masking effect of chitosan. Thus; by increasing the number of epitopes of the OMPs the potential of the outer membrane proteins of *S*. Typhi to stimulate immune response also increased.

Conclusion

The characterization of OMPs alone and with NPCPs as adjuvant results clearly indicates the substantial immunogenic potential and protective immunity induced by OMPs of *S*. Typhi and these can be used as potential vaccine candidates for typhoid. The Vi+ve OMPs don't only activate humoral immunity but also activate several downstream cellular immune responsive pathways resulting in long lasting immunity against *S*. Typhi infection. The total OMP extract is also technically easy to prepare and a mixture of OMPs can also provide multiple antigenic determinants for more effective activation of immune system as compared to recombinant OMPs presenting single antigenic determinant. The low protection of Vi-ve OMPs immunized mice in response to Vi+ve isolates needs further investigations but strengthens the need for more effective and safe vaccine against our local strains.

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Author contributions Yasra Sarwar and Abdul Haque conceived the idea, developed the research, and finalized the manuscript. Ambreen Ayub (first author) performed the experiments, analyzed the data and wrote the manuscript. Yasra Sarwar and Aamir Ali helped in ELISA and animal studies, including sample collection and processing. Qura-tul ain, Asad Bashir and Muhammad Wajid helped in qPCR studies and statistical analysis. Muhammad Usman and Ayesha Ihsan prepared and characterized NPCP for adjuvant preparation. Yasra Sarwar, Abdul Haque and Mazhar Iqbal edited and proof read the manuscript. All authors read and agreed the manuscript for publication.

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Statements and declarations

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References

1. Organization WH (2019) : **Typhoid vaccines**: **WHO position paper**, **March 2018**–**Recommendations**. *Vaccine***37**(2):214–216
2. Yang Y-A, Chong A, Song J (2018) Why is eradicating typhoid fever so challenging: implications for vaccine and therapeutic design. Vaccines 6(3):45
3. Rasheed MK, Hasan SS, Ahmed SI (2019) Extensively drug-resistant typhoid fever in Pakistan. Lancet Infect Dis 19(3):242–243
4. Laghari GS, Hussain Z, Hussain SZM, Kumar H, Uddin SMM, Haq A (2019) : **Antimicrobial susceptibility patterns of Salmonella species in Southern Pakistan**.Cureus**11**(4)
5. Maurya P, Gulati AK, Nath G (2010) Status of Vi gene, its expression and Salmonella pathogenicity island (SPI-7) in Salmonella typhi in India. Southeast Asian J Trop Med Public Health 41(4):913
6. Baker S, Sarwar Y, Aziz H, Haque A, Ali A, Dougan G, Wain J, Haque A (2005) Detection of Vi-negative Salmonella enterica serovar typhi in the peripheral blood of patients with typhoid fever in the Faisalabad region of Pakistan. J Clin Microbiol 43(9):4418–4425
7. Crump JA, Mintz ED (2010) Global trends in typhoid and paratyphoid fever. Clin Infect Dis 50(2):241–246
8. Singh Y, Saxena A, Kumar R, Bhatt P, Saxena M (2017) : **Immunogenic Outer membrane Proteins (Omps) of Salmonella: Potential Candidate for sub-unit vaccine**.Virology & Immunology Journal**1**
9. Rollauer SE, Sooreshjani MA, Noinaj N, Buchanan SK (2015) Outer membrane protein biogenesis in Gram-negative bacteria. Philosophical Trans Royal Soc B: Biol Sci 370(1679):20150023
10. Koebnik R, Locher KP, Van Gelder P (2000) Structure and function of bacterial outer membrane proteins: barrels in a nutshell. Mol Microbiol 37(2):239–253
11. Awate S, Babiuk LAB, Mutwiri G (2013) Mechanisms of action of adjuvants. Front Immunol 4:114
12. El Temsahy MM, El Kerdany ED, Eissa MM, Shalaby TI, Talaat IM, Mogahed NM (2016) The effect of chitosan nanospheres on the immunogenicity of Toxoplasma lysate vaccine in mice. J Parasitic Dis 40(3):611–626
13. Li P, Dai Y-N, Zhang J-P, Wang A-Q, Wei Q (2008) Chitosan-alginate nanoparticles as a novel drug delivery system for nifedipine. Int J biomedical science: IJBS 4(3):221

14. Haque A, Haque A, Sarwar Y, Ali A, Bashir S, Tariq A, Mohsin M (2005) Identification of drug resistance genes in clinical isolates of salmonella typhi for development of diagnostic multiplex pcr. Pak J Med Sci 21(4):402–407
15. Ali A, Haque A, Haque A, Sarwar Y, Mohsin M, Bashir S, Tariq A (2009) Multiplex PCR for differential diagnosis of emerging typhoidal pathogens directly from blood samples. Epidemiol Infect 137(1):102–107
16. Liaquat S, Sarwar Y, Ali A, Haque A, Farooq M, Martinez-Ballesteros I, Laorden L, Garaizar J, Bikandi J (2018) Virulotyping of Salmonella enterica serovar Typhi isolates from Pakistan: Absence of complete SPI-10 in Vi negative isolates. PLoS Negl Trop Dis 12(11):e0006839
17. Hamid N, Jain S (2008) Characterization of an outer membrane protein of Salmonella enterica serovar Typhimurium that confers protection against typhoid. Clin Vaccine Immunol 15(9):1461–1471
18. Laemmli UK (1970) : **Cleavage of structural proteins during the assembly of the head of bacteriophage T4**. *nature***227**(5259):680–685
19. Woranuch S, Yoksan R (2013) Eugenol-loaded chitosan nanoparticles: I. Thermal stability improvement of eugenol through encapsulation. Carbohydr Polym 96(2):578–585
20. Christopoulos T, Diamandis E (1985) 8 assay of albumin in serum based on specific ion-bindingand ion-selective electrode (ISE) potentiometry. Clin Biochem 18(3):202
21. Wollweber LE (1990) **Harlow and D**. **Lane** (**Editors**), **Antibodies**: **A Laboratory Manual**. **XIII + 726 S**., **50 Abb**., **62 Tab**. **Cold Spring Harbor 1988**. **Cold Spring Harbor Laboratory**. **$50.00**. **ISBN**: **0-87969-314-2**. In.: Wiley Online Library;
22. Livak KJ, Schmittgen TD (2001) : **Analysis of relative gene expression data using real-time quantitative PCR and the 2** –**ΔΔCT method**. *methods***25**(4):402–408
23. Kaur A, Kapil A, Elangovan R, Jha S, Kalyanasundaram D (2018) Highly-sensitive detection of Salmonella typhi in clinical blood samples by magnetic nanoparticle-based enrichment and in-situ measurement of isothermal amplification of nucleic acids. PLoS ONE 13(3):e0194817
24. Branca C, D'Angelo G, Crupi C, Khouzami K, Rifici S, Ruello G, Wanderlingh U (2016) Role of the OH and NH vibrational groups in polysaccharide-nanocomposite interactions: A FTIR-ATR study on chitosan and chitosan/clay films. Polymer 99:614–622
25. Gao H-X, He Z, Sun Q, He Q, Zeng W-C (2019) A functional polysaccharide film forming by pectin, chitosan, and tea polyphenols. Carbohydr Polym 215:1–7
26. Indrani DJ, Lukitowati F, Yulizar Y (2017) : **Preparation of chitosan/collagen blend membranes for wound dressing**: **a study on FTIR spectroscopy and mechanical properties**. In: *IOP Conference Series: Materials Science and Engineering*: IOP Publishing: 012020
27. de Lunaa MS, Castaldoa R, Altobellia R, Gioiellaa L, Filipponea G, Gentilec G, Ambrogia V **Chitosan hydrogels embedding hyper-crosslinked polymer particles as reusable broad-spectrum adsorbents for dye removal**
28. Mauricio-Sánchez RA, Salazar R, Luna-Bárcenas JG, Mendoza-Galván A (2018) FTIR spectroscopy studies on the spontaneous neutralization of chitosan acetate films by moisture conditioning. Vib Spectrosc 94:1–6
29. Meiring JE, Shakya M, Khanam F, Voysey M, Phillips MT, Tonks S, Thindwa D, Darton TC, Dongol S, Karkey A (2021) Burden of enteric fever at three urban sites in Africa and Asia: a multicentre population-based study. The Lancet Global Health 9(12):e1688–e1696
30. De Jong HK, Parry CM, van der Poll T, Wiersinga WJ (2012) : **Host–pathogen interaction in invasive salmonellosis**.
31. Pennington SH, Thompson AL, Wright AK, Ferreira DM, Jambo KC, Wright AD, Faragher B, Gilmour JW, Gordon SB, Gordon MA (2016) Oral typhoid vaccination with live-attenuated Salmonella typhi strain Ty21a generates Ty21a-responsive and heterologous influenza virus–responsive CD4 + and CD8 + T cells at the human intestinal mucosa. J Infect Dis 213(11):1809–1819
32. Mittrücker HW, Kaufmann SH (2000) Immune response to infection with Salmonella typhimurium in mice. J Leukoc Biol 67(4):457–463
33. Renu S, Han Y, Dhakal S, Lakshmanappa YS, Ghimire S, Feliciano-Ruiz N, Senapati S, Narasimhan B, Selvaraj R, Renukaradhya GJ (2020) Chitosan-adjuvanted Salmonella subunit nanoparticle vaccine for poultry delivered through drinking water and feed. Carbohydr Polym 243:116434
34. Yüksel S, Pekcan M, Puralı N, Esendağlı G, Tavukçuoğlu E, Rivero-Arredondo V, Ontiveros-Padilla L, Lopez-Macias C, Şenel S (2020) Development and in vitro evaluation of a new adjuvant system containing Salmonella Typhi porins and chitosan. Int J Pharm 578:119129
35. Mathur R, Oh H, Zhang D, Park S-G, Seo J, Koblansky A, Hayden MS, Ghosh S (2012) A mouse model of Salmonella typhi infection. Cell 151(3):590–602
36. Sheikh A, Khanam F, Sayeed MA, Rahman T, Pacek M, Hu Y, Rollins A, Bhuiyan MS, Rollins S, Kalsy A (2011) Interferon-γ and proliferation responses to Salmonella enterica serotype Typhi proteins in patients with S. Typhi bacteremia in Dhaka, Bangladesh. PLoS Negl Trop Dis 5(6):e1193
37. Saxena A, Kumar R, Saxena MK (2017) Vaccination with Salmonella Typhi recombinant outer membrane protein 28 induces humoral but non-protective immune response in rabbit. Veterinary world 10(8):946