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Escherichia coli expressed flagellin C (FliC) of *Salmonella* Typhi improved the protective efficacy of YopE against plague infection

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ABSTRACT

In the current antibiotic resistance scenario, vaccines may provide best defense against lethal bacterial diseases. So far, there is no ideal vaccine available against plague. Despite providing complete protection in small animal models, F1/LcrV based vaccine failed to provide ideal protection in non human primates. Here, we cloned, expressed and purified YopE of *Yersinia pestis* and flagellin C (FliC) of *Salmonella* Typhi. However the best possible protection needs the significant induction of IFN-γ and TNF-α. To determine the protective potential of the recombinant YopE alone or in formulation with FliC, Balb/C mice were immunized subcutaneously. The formulations were prepared with alum, a human compatible adjuvant. In our studies, the combination of YopE + FliC induced significantly strong humoral and cellular immune responses. A combination of YopE + FliC provided 83% protection whereas YopE alone provided only 50% against $100LD_{50}$ of *Y. pestis* in a mouse model.

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1. Introduction

Plague is a highly virulent disease caused by *Yersinia pestis*, a Gram negative, facultative intracellular bacterium [1]. During the 14th century's "Black Death" pandemic, plague has devastated one-third of Europe's population. The human beings usually get the infection through flea bites, soon after the bacteria migrate to nearby lymph nodes. The plague bacilli overcome the innate immune system, grow rapidly and design the swollen buboes, responsible for bubonic plague. If not treated with antibiotics, bubonic plague progresses into septicemic followed by pneumonic form which is 100% lethal, at this stage plague bacilli may be transmitted to healthy individuals through aerosols from infected individuals [2]. In case of primary pneumonic plague, death occurs within 2–6 days of infection, antibiotics are futile and ineffective if not provided within 24 h. Today, plague outbreaks are very rare but there is an utmost need to develop effective countermeasures as antibiotic resistant bacilli have been reported [3]. The genetic makeup of *Y. pestis* is mutable and highly evolving [4]. The development of intentionally aerosolize infectious *Y. pestis* for the possible use as a bio-weapon agent is the matter of concern [2].

Y. pestis bacilli exploit type 3 secretion system (T3SS) for the translocation of virulence factors into the cytosol of target host cells to diminish the cytoplasmic activities and thus overwhelm the host immunity, start pathogenesis and facilitate spreading of bacilli into the host cells [5]. The T3SS is made up of LcrV capped needle composite, translocators and effector Yersinia outer proteins called as Yops *i.e.,* YopH, YopM, YpkA, YopJ, YopK, YopT, and YopE, which are translocated into the cytosol of host cells [6]. Amongst these effector Yops, YopE is conserved in all the pathogenic species of Yersinia and is known as GTPase activating protein (GAP). YopE helps in down regulation of small GTPases *i.e.,* RhoA, Rac1 and cdc42 [7]. Using this mechanism, it blocks phagocytosis, distressing cytoskeletal integrity of the cell and allow bacteria to boom on the surface of macrophages [7,8].

Live attenuated *Y. pestis* vaccine imparts protection in laboratory animals against pneumonic plague [9,10]. These live attenuated vaccines have never been licensed in Europe and United States due to ethical issues however, they are available in some countries [11–13]. Subunit vaccines based on F1/LcrV observed effective in different animal models against all three types of plague [12–15] however; they failed to impart complete protection in African green monkeys [16]. Immune protection correlate assays established that F1/LcrV based subunit vaccines induced strong humoral immune response and provide protection in a mouse model; however the best possible protection needs the significant induction of IFN-γ and TNF-α [17–20]. These conclusions recommend that the new efforts must be directed towards the development of effective subunit vaccines with improved protec-

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tive efficacy and long lasting immunity. In this direction, we evaluated the vaccine potential of recombinant YopE alone and in formulation with FliC of *S.* Typhi as an immunomodulator in a mouse model against plague. In our studies, recombinant FliC from *S.* Typhi significantly enhanced the vaccine potential of YopE in a mouse model against plague.

2. Materials and methods

2.1. Bacterial strains, plasmids, and reagents

DH5α and BL21 (DE3) bacterial cells were purchased from Invitrogen, USA. The pET28a vector was from Novagen, USA. *Yersinia pestis* S1 strain DB182YEPE1 was retrieved from DRDE repository and used for all the conducted experiments. All the challenge experiments were conducted in high containment facility, biosafety level-3 facility at DRDE, Gwalior.

2.2. Cloning and expression of YopE and Flagellin C

The cloning and expression of YopE protein involved in present study have been detailed earlier [21]. In brief, *yopE* was ligated in pET28a vector using *Nde I* and *Sal I* restriction sites and expressed in BL21 (DE3). The gene *fliC* of *S. Typhi* was amplified by PCR using forward 5′-ATACCATGGGCATGGCACAAGTCATTAATACAAACAG-3′and reverse primers 5′-ATACTCGAGACGCAGTAAAGAGAGGACGTTTTG-3′. The amplicon was ligated in pET28a vector using the *Nco I* and *Xho I* restriction sites (underlined nucleotide sequence). Recombinant FliC was expressed in BL21 (DE3) after induction with 1 mM IPTG. The expression of recombinant YopE of *Y. pestis* and FliC of *S. Typhi* was confirmed by Western blot using anti-histidine antibody after SDS-PAGE.

2.3. Purification of YopE and Flagellin C

The recombinant proteins FliC and YopE carrying histidine tag at C- and N-terminus respectively were purified by immobilized metal affinity chromatography (IMAC) using Ni-NTA column (Qiagen, Germany). Recombinant FliC protein was purified using native condition whereas YopE was purified under denaturing conditions using 8 M urea. The purified proteins were dialysed and estimated by BCA kit (Sigma, USA). The endotoxin levels were estimated using Limulus Amoebocyte Lysates (LAL) QCL-1000 kit (Cambrex Biosciences, USA) using the protocol provided in the kit.

2.4. Immunization of animals

BALB/c mice (female, 6–8 weeks old) were taken from animal house of DRDE. The animals were housed in accordance with recommendations of committee for the purpose of control and supervision of experiments on animals, Govt. of India. The study had the approval of Institutional Animal Ethics Committee and had a protocol number MB-24/53/SKV. All live *Y. pestis* cultures and animal challenge experiments were conducted in BSL-3 facility. The mice were divided into two different batches, each batch was divided into four groups (6 mice/group) *i.e.,* Control group; FliC group; YopE group and FliC + YopE group. All the mice were immunized subcutaneously on ventral thoracic portion with 15 μg/mouse of each purified protein in formulation with aluminum hydroxide gel. Control group mice were injected with PBS only. The immunizations were given on day 0; day 14; and day 21. Blood was collected from all the immunized animals on day 0, 21, and 28 as shown in Fig. 1c [B]. Animals of batch I were used for challenge studies and batch II were used for cytokine profiling.

2.5. IgG titer

Endpoint titers of IgG antibody were determined by ELISA in the serum samples collected from immunized animal groups after first and second boosters. Endpoint titers of IgG were targeted against YopE and FliC. The 96 well ELISA plates were coated individually with recombinant YopE and FliC antigens (100 ng/well each antigen) diluted in carbonate buffer [50 mM, pH 9.6]. Initially, plates were incubated at 37 °C for 1 h and thereafter incubated at 4 °C for overnight. Next day, plates were washed thrice with 0.05% Tween 20 in PBS (PBS-T), blocked with 3% bovine serum albumin (BSA) and incubated for 2 h at 37 °C. Plates were washed, test sera were serially diluted in triplicate wells (100 μl/well) from immunized and control mice collected after first and second boosters. Each serum sample was taken in triplicate. The plates were incubated for 1 h at 37 °C and followed by washings. ELISA plates were further incubated with horseradish peroxidase labelled rabbit anti-mouse IgG (Sigma, USA) at 1:20,000 dilutions for 1 h at 37 °C. After extensive washings, *o*-phenylenediamine dihydrochloride (OPD) was added to develop the reaction for 10 min. The reaction was stopped by 2 N H_2SO_4 and OD_{490} was read by a multimode reader (Biotek, USA).

2.6. Cytokine profile

Spleens were removed in aseptic conditions from batch-II animals and spleen cells were prepared. The splenocytes were counted and 1×10^6 cells/well from each group were poured in triplicate wells of a culture plate. The spleen cells were stimulated with YopE or FliC vaccine antigens or ConA (5 μg/ml each). The cells were grown in a CO_2 incubator for 48 h, all the supernatants were collected from the cells. To measure the expression levels of cytokines for each group, the samples were taken in triplicates. The concentrations of TNF-α and IFN-γ were determined using kits (BD Biosciences, USA) according to the manufacturer's protocols. The expression levels of TNF-α and IFN-γ were estimated in pg/ml with the help of standard curves.

2.7. Challenge of animals

The virulent strain (S1) of *Y. pestis* was grown on BHI agar plate from the stock. Further, a single colony was picked up from BHI agar plate and inoculated in 5 ml of BHI broth. The culture was grown at 28 °C for 48 h in a shaker incubator and the colonies (CFU/ml) were counted on BHI agar plate. In order to test the protective efficacy of recombinant YopE as alone or in combination with FliC, after 1 month of last booster, all the immunized and control animals were experimentally infected [14,21,22]. In brief, the colonies $1x10^5$ CFU/100 μl (100 LD_{50}) of *Y. pestis* were adjusted in sterile PBS. The animal groups were administered 1×10^5 CFU/-mouse of *Y. pestis* by subcutaneous route. All the mice were observed for their survival for 30 days of post challenge as shown in Fig. 1c [B].

2.8. Statistical analysis

Results presented here as the mean value ± standard deviation (SD). All the statistical comparisons were done by GraphPad Prism software, one way ANOVA, All Pairwise Multiple Comparison Procedure (Fisher LSD Method). $^{**}P < 0.001$; $^{***}P < 0.0001$. The graphs for animal survival were made by Kaplan–Meier method using GraphPad Prism software 6.0 ($^{****}P < 0.0001$).

3. Results

3.1. Cloning of yopE of Y. Pestis and fliC of S. Typhi genes in pET vector

Cloning and expression of recombinant YopE involved in this study has been described earlier [21]. Briefly, the gene *yopE* (660 bp) of *Y. pestis* encoding YopE of 23 kDa protein was cloned in pET28a vector and expressed in BL21 (DE-3). The gene *fliC* of 1521 bp from S. *Typhi* encoding FliC of 55 kDa recombinant protein was cloned in pET28a vector. The in-frame of ligated *fliC* gene was confirmed by nucleotide sequencing. The ligated gene product corresponding to FliC was transformed into chemically competent BL-21(DE3) cells. The positive clones were screened on LB agar plates containing kanamycin.

3.2. Expression and purification of recombinant FliC and YopE proteins

Positive clones were grown and induced with IPTG for the identification of clones able to express the protein of interest. The expression profile of recombinant proteins FliC and YopE were performed by SDS-PAGE. The SDS-PAGE profiles of uninduced and induced cultures for FliC and YopE have been shown in Figs. 1a [A] and 1b [A] respectively. Western blot analysis with anti-His-tag antibody detected a 55 kDa protein band [Fig. 1aB] and 23 kDa protein band [Fig. 1bB] in the induced bacterial lysate, representing the recombinant FliC and YopE respectively, but the same bands were not observed in the uninduced bacterial lysates (Figs. 1a and 1b).

To purify the recombinant proteins, the in-frames of FliC and YopE were facilitated to carry the 6X-histidine tags at C-terminus and N-terminus respectively. The FliC was purified under native conditions however YopE was purified under denaturing conditions using 8 M urea by Ni-NTA affinity chromatography. The endotoxin level was detected less than 5 endotoxin unit (EU)/25 μg of each purified protein. Elutes of purified recombinant FliC and YopE proteins were analysed by SDS-PAGE as shown in Figs. 1a [C] and 1b [C]. Concentrations of purified FliC and YopE were estimated and the obtained yields were 15 and 20 mg/L of shake flask cultures respectively. After dialysis and estimation, both purified recombinant FliC and YopE proteins were analysed on SDS-PAGE as shown in Fig. 1c [A].

3.3. Humoral immune response

In order to test the humoral immune response evoked by YopE alone or in combination with FliC, IgG endpoint titers were determined by ELISA in the sera taken seven days after first and second boosters respectively. The cut-off value for ELISA was determined as the mean OD (+2 SD) from control sera performed at 1:200 dilution. IgG endpoint titers were analysed as reciprocal of the highest

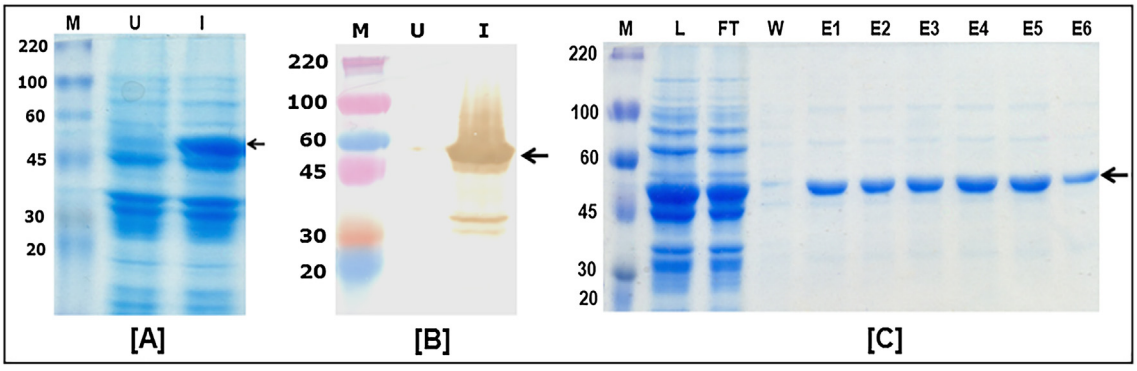

Fig. 1a. Expression, Western blot and purification profile of recombinant FliC protein of *S.* Typhi: [A] SDS-PAGE profile of recombinant FliC protein expression: Lane M- Pre-stained protein marker; Lane U- Uninduced *E. coli* cell lysate; Lane I- Induced *E. coli* cell lysate. The arrows at the right of the profiles side indicate the position of FliC protein of 55 kDa. [B] Western blot profile of recombinant FliC protein probed with anti-histidine tag antibody. Lane M- Pre-stained protein marker; Lane U- Uninduced *E. coli* cell lysate; Lane I- Induced *E. coli* cell lysate. [C] Purification profile of recombinant FliC: Lane M- Protein marker; Lane L- Pre-load; Lane FT- Flow through; Lane W- Wash; Lane E1-E6- Eluted fractions of purified recombinant FliC.

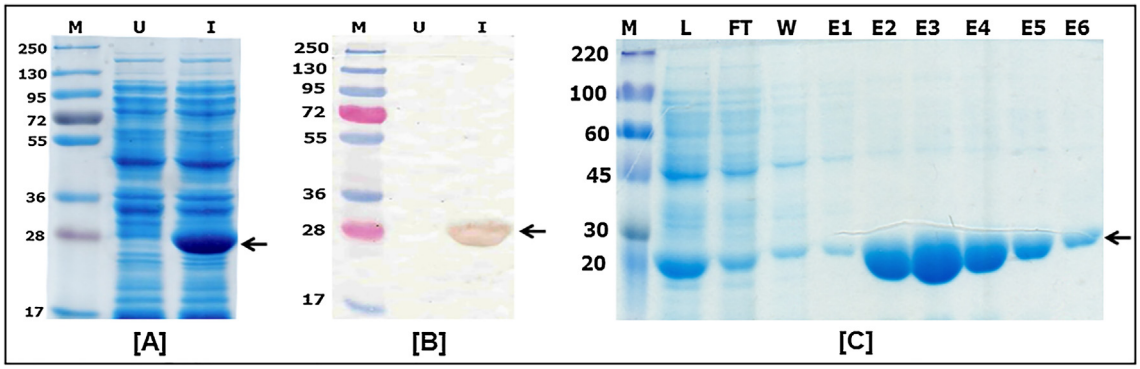

Fig. 1b. Expression, Western blot and purification profile of recombinant YopE protein of *Y. pestis*: [A] SDS-PAGE profile of YopE expression: Lane M- Pre-stained protein marker; Lane U- Uninduced *E. coli* cell lysate; Lane I- Induced *E. coli* cell lysate. The arrows at the right of the profiles side indicate the position of YopE protein of 23 kDa. [B] Western blot profile of YopE protein probed with anti-histidine tag antibody. Lane M- Pre-stained protein marker; Lane U- Uninduced *E. coli* cell lysate; Lane I- Induced *E. coli* cell lysate. [C] Purification profile of recombinant YopE: Lane M- Protein marker; Lane L- Pre-load; Lane FT- Flow through; Lane W- Wash; Lane E1-E6- Eluted fractions of purified recombinant FliC.

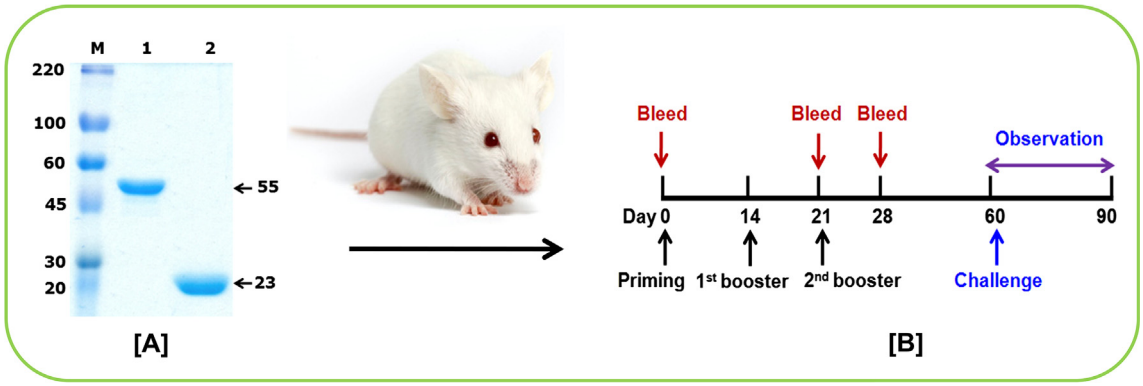

Fig. 1c. Vaccine antigens and the schedule of animal immunization, blood collection and challenge experiments: [A] SDS-PAGE profile of purified vaccine candidates; Lane M- Pre-stained protein molecular weight marker; Lane 1- Purified recombinant FliC protein; Lane 2- Purified recombinant YopE. [B] Schematic representation of vaccination schedule, blood collection and challenge experiments.

serum dilution showing an OD > cut-off value. IgG endpoint titer to YopE was observed 32,000 in the sera collected from YopE group whereas it was 64,000 from FliC + YopE group after first booster. IgG endpoint titer to YopE after second booster was 64,000 in the sera collected from YopE group and 256,000 from FliC + YopE group. There was a significant difference in the IgG endpoint titers in the sera collected after first booster ($^{**}P < 0.001$) and second booster ($^{***}P < 0.0001$) from FliC + YopE immunized animals in comparison to YopE immunized animals [Fig. 2b (a)].

3.4. Cellular immune response

The induced levels of IFN-γ, and TNF-α in the collected supernatants of splenocytes were determined. There was a significance difference ($^{***}P < 0.0001$) in the expression levels IFN-γ [Fig. 2a (a)], and TNF-α [Fig. 2a (b)] in YopE + FliC vaccinated animals in comparison to control group. ConA was used to induce the spleen cells of all the groups and it responded non-specifically. There was a significant difference ($^{***}P < 0.0001$; $^{**}P < 0.001$) in the concentration of IFN-γ and TNF-α in YopE + FliC vaccinated group in comparison to YopE or FliC immunized groups.

3.5. Protective potential

To test the protective efficacy and vaccine potential of YopE alone or in combination with FliC as an immunomodulator, the vaccinated animals were challenged with 100 LD_{50} of *Y. pestis* (S1 strain). All the challenged animals were monitored for their survival for 30 days post infection [Fig. 2b (b)]. YopE in combination with FliC imparted 83% protection ($^{****}P < 0.0001$) whereas YopE alone provided 50% protection only. No protection was observed in control and FliC groups.

4. Discussion

There is no licensed vaccine against plague so far. It is due to the stability problems, poor and inconsistent immunogenicity and associated manufacturing problems. To conquer these troubles, there is an utmost need to develop new or innovative ideas to design novel immunogens. These new vaccine proposals would be of immense interest to develop effective vaccines against biothreat agents.

Mostly, subunit vaccines are safe but poorly immunogenic in nature in comparison to whole cell based vaccines; therefore, molecular adjuvants are essential for improving vaccine potential by modulating the immune responses to the vaccine antigens. Towards developing an efficacious plague subunit vaccine, we have cloned, expressed and purified FliC of *S.* Typhi for the evaluation of vaccine potential of recombinant YopE alone and in combination with FliC against plague infection in a mouse model. Flagellin, a structural protein of the flagellar filament of *S.* Typhi has shown remarkable potential as an adjuvant, either as a fusion protein or by co-administration with vaccine antigens [19,23–25]. Flagellin is a strong inducer for both the innate and adaptive immunity. It

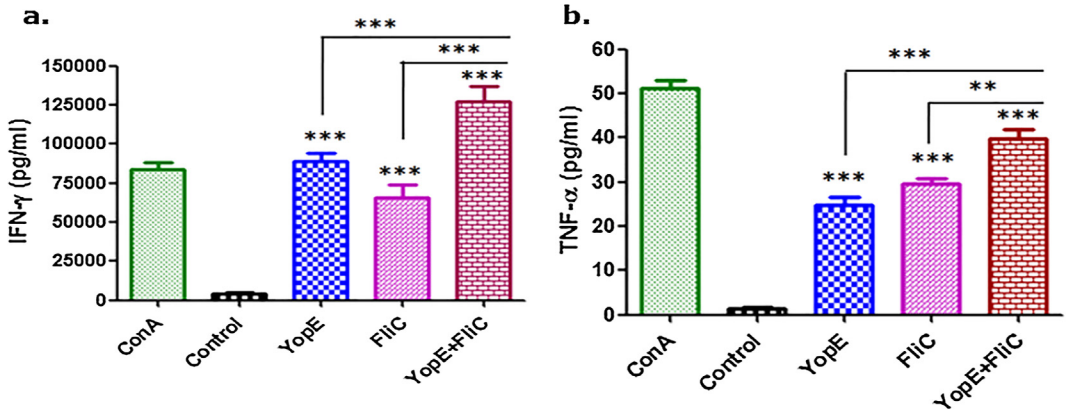

Fig. 2a. Cytokine profile of immunized mice. Spleen cells were prepared from all the vaccinated and control groups. The cells were induced with vaccine antigens *i.e.*, YopE and FliC (5 μg/ml each) and grown for 48 h. The induced levels of IFN-Y (a) and TNF-α (b) were measured in pg/ml as represented in graphs. Here, we represented the data of 6 mice of each group ± SD. All the statistical comparisons were done by GraphPad Prism software, one way ANOVA, $^{**}P < 0.001$; $^{***}P < 0.0001$.

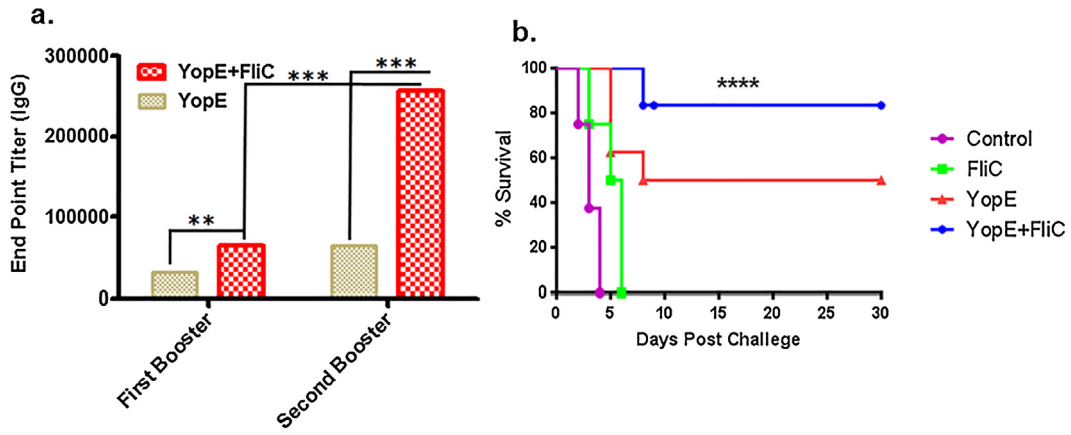

Fig. 2b. Humoral immune response and protective potential: (a) End point titers of IgG antibody were determined by ELISA in the serum samples collected from immunized animal groups after first and second boosters. Statistical analysis was done by one way ANOVA, all Pairwise Multiple Comparison Procedure (Fisher LSD Method) $^{**}P < 0.001$; $^{***}P < 0.0001$. (b) The Immunized and control group animals were challenged with100 LD_{50} of *Y. pestis* (S1 strain). The animals were examined for 30 days after challenge. The survival curve was determined by Kaplan Meier's method using GraphPad Prism software 6.0 ($^{****}P < 0.0001$).

is documented that the required amount of flagellin to evoke a maximal antigen-derived humoral immune response is usually lower in comparison to required dose to induce maximal innate immunity [26,27]. Flagellin triggers the cells through pattern recognition receptor TLR5 [28,29]. TLR5 is present on immune and non-immune cells *i.e.,* natural killer (NK) cells, macrophages, neutrophils, monocytes, lymphocytes, dendritic cells (DCs) and epithelial cells [30,31]. Flagellin or flagellin-based vaccine triggers these cells to lead the stimulation of chemokines and cytokines that encourage a marked recruitment in B and T cells to draining lymph nodes [26,32] and therefore, enhances the capability of antigen-specific lymphocytes to counteract the pathogens. These rationales practically add to the overall effectiveness of flagellin-based vaccine formulation.

In the present study, we have cloned, expressed and purified recombinant FliC for immunomodulating the vaccine potential of YopE against plague. The humoral and cellular immune responses of a combination (YopE + FliC) were evaluated in comparison to YopE alone in Balb/C mice. Recombinant FliC significantly modulated the IgG response in the YopE + FliC vaccinated animal group in comparison to YopE alone. There was significant difference in the induction of IFN-γ and TNF-α in YopE + FliC immunized animal group in comparison to YopE alone. FliC significantly enhanced the protection (83%) in YopE + FliC vaccinated animal group whereas YopE alone provided 50% protection against 100LD_{50} of *Y. pestis* infection. In these studies, bacterially expressed recombinant YopE in formulation with flagellin C could provide only 83% protection against plague. Despite significant modulation of humoral immune response, cellular immune response and protective potential, this vaccine formulation FliC + YopE got fail to achieve the goal. In this study, YopE in formulation with FliC of *S.* Typhi could not provide 100% protection in Balb/C mice against plague. Our further studies are in progress to achieve 100% protection using YopE in combination with other immunodominant antigens of *Y. pestis* or by applying other clinically approved new age adjuvants.

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