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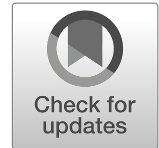

Functional characterization of a broad and potent neutralizing monoclonal antibody directed against outer membrane protein (OMP) of *Salmonella typhimurium*

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Abstract

In the present study, we have generated a murine monoclonal antibody (mAb) named Sal-06 by using the crude outer membrane protein preparation of *Salmonella enteric* subsp. *enterica serovar Typhimurium* ATCC 14028 strain as antigen. Sal-06mAb belonging to IgG1 isotype demonstrated broad cross-reactivity to standard and isolated strains of genus *Salmonella* and others such as *Escherichia coli*, *Klebsiella pneumonia*, and *Proteus mirabilis*. Cross-reactivity across several bacterial genera indicated that the epitopes reactive to Sal-06mAb are conserved among these members. Neutralizing effects of Sal-06mAb on *Salmonella* growth and survival was evaluated in vitro using bacteriostatic and bactericidal activity with and without complement and bacterial invasion inhibition assay. Sal-06mAb demonstrated a bacteriostatic effect on the growth of *S. typhimurium* ATCC 14028 strain which is both time and concentration (of mAb) dependent. It was also found that the bacterial growth inhibition was complement independent. When the bacterial cells were preincubated with Sal-06mAb, it reduced the adherence and invasion of bacterial cells into A549 epithelial cell line. This was confirmed by CFU count analysis, phase contrast, and fluorescence microscopy. Scanning electron microscope (SEM) imaging confirmed the antimicrobial effects of Sal-06mAb on *S. typhimurium* ATCC 14028. The development of broadly reactive and cross protective Sal-06mAb opens new possibilities for immunotherapy of sepsis caused by Gram-negative *Enterobacteriaceae* members.

Keywords Monoclonal antibody · Neutralizing antibody · Cross-reactivity · Enterobacteriaceae · Bacteriostatic · Bactericidal assay

Introduction

Salmonella is an important genus of the family *Enterobacteriaceae* and the most common pathogenic bacteria which causes diarrhea, gastroenteritis, typhoid, paratyphoid fever, septicemia, and other clinical syndromes with varying degrees of severity (Di Febo et al. 2019; Li et al. 2019). *Salmonella* is commonly found in intestinal tract of humans and animals and therefore presence of *Salmonella* in food and raw materials is an indication of fecal contamination. Certain non-typhoidal *Salmonella* strains are responsible for bloodstream infections which are referred to as invasive non-typhoidal *Salmonella* (INTS) infections. The Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) published that *Salmonella* enterocolitis resulted in ~ 95.1 million cases and 50,771 deaths in 2017. The symptoms are not typical of diarrhea and present as febrile illness with higher fatality rate (Stanaway et al. 2019). The disease burden is disproportionate affecting adults or children with weak immune system. Intervention and control of the infection depends on rapid detection of these pathogens followed by appropriate therapeutic interventions.

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Antimicrobial resistance to several classes of antibiotics such as penicillins, tetracyclines, fluoroquinolones, sulfonamides, aminoglycosides, and cephalosporins is another major concern in treatment of *Salmonella* infections (Lamas et al. 2018). Passive immunization has been successfully used as an alternate method of prophylaxis against several gastrointestinal pathogens such as *Campylobacter*, *Salmonella*, *E. coli*, rotavirus, and coronavirus (Esmailnejad et al. 2019). Interest towards antibody-based therapy was prompted based on the need to find suitable alternatives to conventional antibiotics in view of a widespread antimicrobial resistance.

Selection of right virulence factors as target for therapeutic intervention plays a key role in disease control. Several studies have shown that outer membrane proteins (OMPs) of bacteria are strong immunogenic components and possible vaccine candidates (Babu et al. 2017; Abadi et al. 2018; Neema and Karunasagar 2008). Outer membrane proteins include integral membrane proteins as well as lipoproteins that are anchored to the outer membrane via N terminally attached lipid. Generally, OMPs are characterized by β-barrel structures and integral OMPs are essential for maintaining the integrity and selective permeability of bacterial membranes (Lin et al. 2002). Some of them serve as adhesins and play an important role in virulence (Ebanks et al. 2005; Khushiramani et al. 2008). Outer membrane proteins are extremely immunogenic (Li et al. 2014) and therefore they would be effective candidates for vaccine and diagnostic development owing to their exposed epitopes on the cell surface. The OMPs of *Salmonella* are also known to have a significant role in eliciting immune response (Meenakshi et al. 1999). They have been considered potential candidates for conferring protection against typhoid. *Salmonella* OMPs have been investigated as potential vaccine candidates, virulence factors, and diagnostic antigens (Isibasi et al. 1988). Therefore, generation of antibodies specific against surface and structural components might prevent establishment of infection.

In the present study, we utilized the *S. typhimurium* OMP preparation as antigen of choice to develop mAbs reactive against the members of the genus *Salmonella*. One monoclonal antibody namely Sal-06 was functionally characterized by several in vitro assays to study the effectiveness of mAb in fighting the *Salmonella* infections. A preliminary attempt was made to understand the ability of mAb to block the pathogen before undertaking in vivo challenge studies. The cross-reactivity, antibacterial and invasion inhibition assays were performed to examine the anti-*Salmonella* properties of Sal-06 monoclonal antibody.

Materials and methods

Materials

Bacterial cultures and cell lines

The bacterial strains used in the present study are *S. typhimurium* ATCC 14028, *S. paratyphi* MTCC 735, *S. typhi* Typhi isolate Gwalior, *S. bovismorbificans* MTCC 1162, *S. infantis* MTCC 1167, *S. newporti isolate*, *S. typhimurium* NCIM 5278, *S. enteritidis* isolate Gwalior, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* MTCC 3310, *Citrobacter freundii* ATCC 8090, *Shigella flexneri* ATCC 9199, *Enterobacter aerogenes* ATCC 13048, and *Escherichia coli* ATCC 10536. A549 epithelial cell line was used in the present study. Cell line A549 was purchased from National Centre for Cell Sciences (NCCS), Pune, India. A549 cell line is derived from adenocarcinomic human alveolar basal epithelial cell.

Media components, chemicals, and reagents

Dehydrated media such as brain heart infusion broth and Mueller-Hinton broth for bacterial propagation were procured from HiMedia laboratories, Mumbai, India. Cell culture media, reagents, and antibiotics were procured from Sigma-Aldrich, India. Inorganic salts and organic solvents were from Sisco Research Labs, India. BHI broth or agar was used for culturing, propagation, and storing of all bacterial strains. Mueller-Hinton media was used for bacteriostatic/bactericidal assays and invasion-inhibition assays. All bacterial cultures used in this study were grown at 37 °C under constant agitation of 200 RPM. A549 cell line was propagated in Dulbecco Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) with appropriate antibiotics and maintained at 37 °C under 5% CO_2.

Outer membrane protein preparation

Crude OMP was prepared from overnight culture of *S. typhimurium* ATCC 14028 as described by Arora et al. (2006). Briefly, *S. typhimurium* was grown in Brain Heart infusion broth overnight followed by harvesting the cells by centrifugation at 4 °C. The cells were washed in PBS twice and dissolved in 30 ml of 10 mM Tris containing 0.3% NaCl. The cells were subjected to sonication (Vibra-Cell sonicator, Sonics & Materials Inc., Newtown, CT), at amplitude 10 μm in ice for 10 s of six repetitions with 20-s intervals. The lysate was subjected to centrifugation for 2 min at 7000*g* followed by centrifugation of supernatant at 14,000*g* for 1 h at 4 °C. The pellet was suspended in 10 mM Tris containing 3% sodium lauryl sarcosinate (sarkosyl) and incubated overnight at

4 °C. Crude OMP extract was obtained by centrifugation at 14,000*g* at 4 °C. OMPs were washed in distilled water and dissolved in 1× PBS and stored at − 20 °C for further use. The protein concentration was determined by Lowry's colorimetric assay.

Generation and characterization of Sal-06 monoclonal antibody

Hybridoma procedure

Outer membrane protein preparation extracted from strain *S. typhimurium* ATCC 14028 was used for immunizing 5–6-week-old female BALB/c mice. Mice were maintained in 12-h light and dark cycles alternately. Mice were provided with food pellets from a commercial vendor. Mineral water from Kent water purifier was given to mice during the study period. Rice hulls were used as bedding material and changed every 2 or 3 days. Two female mice were housed in each polypropylene enclosure. Fifty microgram quantity of OMP was administered subcutaneously in emulsion with Freund's complete adjuvant followed by two booster doses with the same quantity of protein in emulsion with Freund's incomplete adjuvant at 15-day interval. Antibody titers were determined by indirect ELISA using extracted OMP preparation as antigen. After sufficient titers were reached, mice were sacrificed, spleens were aseptically dissected, and the collected splenocytes were fused with mouse myeloma cell line SP2/O Ag-14 by PEGylation method. The fused cells were propagated in HAT medium and the hybrids were screened for reactivity with OMP preparation. One mAb named Sal-06 showing strong reactivity was separated and used for further studies. Sal-06mAb was purified by growing Sal-06 clone in DMEM supplemented with 10% FBS in 75 cm^2 culture flasks until 75% confluency. The cells are transferred to serum-free media and grown for another 2–3 days. The cell-free culture supernatant was separated and used for purifying Sal-06mAb.

Ammonium sulfate precipitation and dialysis

Cell culture supernatant (CCS) collected from Sal-06 clone was subjected to centrifugation at 1100*g* for 10 min to remove cells. The supernatant (100 ml) was treated by ammonium sulfate precipitation for concentration and recovery of mAbs. Briefly, to the CCS, $1/10^{th}$ volume 1 M Tris-Cl (pH 8.0) was added and mixed. To this mixture, 100% ammonium sulfate solution was added dropwise under continuous stirring until the mixture turned turbid (required about ~ 30 ml). The mixture was incubated at 4 °C for 2 h. The solution was subjected to centrifugation at 6000*g* at 4 °C for 1 h. To the pellet, 5 ml of 100% ammonium sulfate solution was added, mixed, and again subjected to centrifugation at 6000*g* at 4 °C for 30 min. The pellet was dissolved in 2.0 ml of 1× PBS and the excess salt was removed by dialysis against 1000 volumes of 1× PBS solution overnight. The buffer was changed at least three during dialysis before collecting the concentrated mAb from Sal-06 clone. Finally, the Sal-06mAb was purified to homogeneity using protein A chromatography kit (Sigma-Aldrich, India). Isotype of Sal-06mAb was determined by Isoquick mouse monoclonal antibody isotyping kit (Sigma-Aldrich, India).

Lyophilization

Concentrated Sal-06mAb was divided into aliquots and subjected to lyophilization for future use. Briefly, aliquots were frozen in − 80 °C and the frozen samples were placed in a lyophilizer (Lyodel – Delvac pumps, India) precooled to − 55 °C and subjected to lyophilization at 1.2 psi for 4 h. The lyophilized samples were stored in − 20 °C until further use. The completely dried samples were dissolved in sterile distilled water when required and passed through 0.22-μm sterile syringe filter (Pall Life Sciences, India). The antibody concentration was quantified by Lowry's colorimetric assay using BSA as standard. The concentration of the mAb was adjusted to ~ 2 mg/ml.

SDS-PAGE and western blot analysis

SDS-PAGE and western blot analysis were carried out to check the reactivity of Sal-06mAb with different bacterial strains belonging to *Enterobacteriaceae*. Briefly, SDS-PAGE was performed using Bio-Rad Mini-PROTEAN Tetra Cell apparatus as per the procedure of Laemmli (1970) with minor modifications. One milliliter of overnight bacterial cells was harvested by centrifugation and dissolved in equal volumes of 1× PBS and 2× Laemmli buffer. The bacterial samples were subjected to boiling for 10 min followed by centrifugation at 13,500*g* for 5 min to separate cell debris. Ten microliters of cell lysate supernatant was loaded on 12% SDS-PAGE gels and resolved at 100 V. Separated proteins on resolved gels were transferred to charged nitrocellulose membrane (0.45 μm, Pall Life Sciences) by electroblotting by wet transfer in Bio-Rad Mini Trans-Blot cell at 70 V for 60 min. After blotting, membranes were blocked in 5% skim milk solution overnight at 4 °C. Excess milk protein was washed in PBST (Tween 20, 0.05%) solution. Next, the membrane was incubated in 1:2000 dilutions of Sal-06mAb at room temperature on a gel rocker. The membrane was washed in PBST and incubated with goat anti-mouse IgG conjugated with HRP (1:5000 dilutions). After washing the membrane with PBST, the blots were developed using diaminobenzidine tetrahydrochloride hydrate and 0.003% H_2O_2 solution in PBS.

Dot ELISA

Overnight cultures were used for dot ELISA. Five hundred microliter volume of culture was harvested by centrifugation followed by dissolving the bacterial cells in equal volumes of carbonate-bicarbonate buffer (pH 9.2). Ten microliters of the cells was coated onto grids of 1 × 1 cm on nitrocellulose (NC) strip and air dried for 10 min. NC strips were blocked in 5% milk solution for 1 h at room temperature. Strips were washed in PBST solution 4–5 times and incubated in diluted Sal-06mAb. After washing, membrane was incubated with goat anti-primary antibody conjugated to HRP. Membranes were further washed in PBST and developed with diaminobenzidine (DAB) tetrahydrochloride (Sigma-Aldrich, India) solution and 0.003% H_2O_2 in PBS.

Bacteriostatic/bactericidal studies of Sal-06mAbSal-06mAb

The effect of Sal-06mAb on the survival and growth of *S. typhimurium* ATCC 14028 was evaluated by bacteriostatic/bactericidal assays using microtiter plates. *S. typhimurium* ATCC 14028 strain was grown overnight in Mueller-Hinton broth (MHB). The culture was diluted to 10^5 CFU/ml in MHB. Lyophilized Sal-06mAbSal-06mAb was reconstituted in sterile distilled water and adjusted the concentration to 1 mg/ml. The mAb was added to *Salmonella* culture at various concentrations such as 120, 60, and 30 μg. The mAb was added to MHB (100 μl) and mixed with 100 μl of *S. typhimurium* ATCC 14028 diluted in the above step in triplicates in 96-well microtiter plate with lid. As control, sterile MHB with no mAb and 100 μl of bacterial cells were used. To understand if the mAb would non-specifically bind to other bacteria and inhibit the growth, we also mixed *Enterobacter aerogenes* ATCC 13048 in triplicates with 120 μg of mAb. Wells with 200 μl of sterile MHB were used as blank. The plate was incubated at 37 °C with constant shaking at 200 RPM in Tecan Multimode reader (M200Pro) and the absorbance was read at 595 nm every hour for 14 h.

Effect of complement on bactericidal activity

The effect of complement on bactericidal activity was studied by adding mouse complement from serum. The blood was collected from unimmunized mice by retro-orbital puncturing. The blood was incubated at 37 °C for 1 h and followed by centrifugation at 2348*g* for 5 min to separate the serum. The serum was collected and stored at − 20 °C until further use. The complement assay was performed similar to bacteriostatic assay except that 10 μl of serum which contained complement was added to each well along with mAb, MHB, and *S. typhimurium* ATCC 14028 cells as described in the methods section "Bacteriostatic/bactericidal studies of Sal-06mAb." The microtiter plate was covered and kept under shaking at 175 RPM for 14 h and the absorbance was read at 595 nm every 1 h.

Invasion inhibition assay

Invasion inhibition assay was performed on A549, a human alveolar epithelial cell line. The cells were grown in tissue culture flask to 80–90% confluence in DMEM with 10% FBS and suitable antibiotics at 37 °C with 5% CO_2. Cells were dislodged by 0.025% trypsin treatment. The cells were centrifuged at 300*g* at room temperature for 5 min. The cell pellet was resuspended in fresh DMEM with 10% FBS and transferred to 24-well plates and incubated at 37 °C overnight. The following day, the culture supernatant was removed from the A549 cells and washed with sterile 1× PBS two times. At the same time, the bacterial cells from overnight culture were harvested and washed in PBS, and cells were diluted to 1 × 10^5 CFU/ml in DMEM with 10% FBS. Similarly, mAb was diluted to 100 μg/ml in DMEM with 10% FBS. Now, equal volumes (300 μl) of bacterial culture and mAb were mixed in a single sterile micro centrifuged tube and incubated at 37 °C for 30 min. As control, only bacterial cells without mAb were used. This mixture was added along with an equal amount of DMEM media supplemented with 10% FBS to A549 cells. The plate was incubated in CO_2 incubator at 37 °C and 5% CO_2 for 1 h. After incubation, the media was removed and washed with sterile 1× PBS twice. Two hundred microliters of fresh DMEM media containing gentamicin was added and incubated at 37 °C for 30 min. The media was removed, and cells were washed with sterile 1× PBS twice. A549 cells were lysed with 100 μl of 1% triton X-100. Cells were observed under microscope for confirmation of lysis. To the lysed cells, 900 μl of sterile PBS was added, mixed, and collected aseptically in sterile micro centrifuge tubes. One hundred microliters of above culture suspension was subjected to pour plating on plate count agar along with appropriate controls. The plates were incubated at 37 °C for 24 h and colonies were counted.

Microscopy studies

A549 cells were seeded as described in the methods section "Invasion inhibition assay" into 12-well plates containing gelatin-coated glass cover slips. The cells were incubated at 37 °C for 12 h in 5% CO_2. Then, they were washed with sterile 1× PBS or DMEM media. The mixture of *S. typhimurium* ATCC 14028 culture and mAb as described in the methods section "Invasion inhibition assay" was added to the 2 wells as duplicates and *S. typhimurium* ATCC 14028 cells was added without mAb as control. The bacterial cells were allowed to adhere for 2 h at 37 °C in 5% CO_2. Unattached bacterial cells were removed by washing 5–6 times with 1 ml pre-warmed sterile 1× PBS. Five hundred microliters of diluted FITC

secondary conjugate was added to each well and kept for 2 h at room temperature in a dark place. Again, the wells were washed with sterile 1× PBS several times. The cells on the coverslips were fixed by dipping them in 4% formalin and then dried for observation under phase contrast and fluorescent microscope.

Scanning electron microscopy

The effect of mAb on live *Salmonella* cells' morphology was observed by a scanning electron microscope (FEI, model no.: Quanta 200) analysis. Briefly, *S. typhimurium* ATCC 14028 was grown overnight in BHI broth. Bacterial cells were washed in filter-sterilized PBS and adjusted the cell count to 10^6 CFU/ml. Hundred microliters of cells were mixed with 50 μg of mAb and incubated at 37 °C for 1 h. Bacterial cells without mAb addition were used as controls. Monoclonal antibody treated and untreated bacterial cells were fixed on conducting dual side carbon tape and coated with gold using a sputter coater (Emitech, model no.: SC7620) and dried out in CO_2 chamber. The changes in surface morphological structure of bacterial cells were captured in an environmental mode under SEM at a magnification of × 10,000.

Statistical analysis

All the statistical analyses were performed using Microsoft Excel 2007. All the ELISA experiments were done in triplicates and the data was represented as Mean ± SD. Statistical difference between different groups was analyzed using univariate ANOVA and the differences at $p < 0.05$ were considered to be significant.

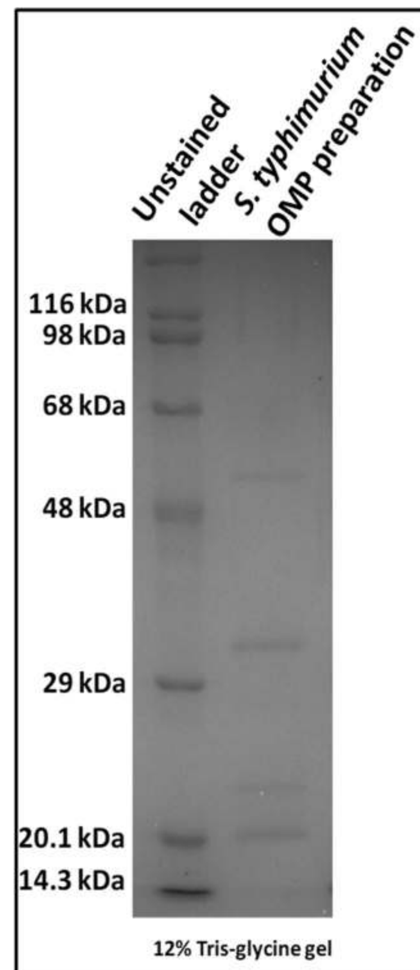

Fig. 1 Coomassie-stained SDS-PAGE gel showing protein profile of crude outer membrane protein prepared from *S. typhimurium* ATCC 14028 strain

Results

Outer membrane protein preparation and immunization

Separation of membrane proteins was achieved using sarkosyl. This method was found to be optimal in recovering of OMPs from membranes (Ghatak et al. 2003). Separation of extracted OMPs showed several bands on Coomassie brilliant blue-stained SDS-PAGE gels ranging from 15 to 70 kDa (Fig. 1). The OMP concentration was found to be ~2.5 mg/ml by Lowry's assay. The concentration was adjusted to 1 mg/ml using PBS as diluent. Outer membrane proteins were directly administered as antigen in mice. Antibody titer increased progressively with each booster dose with crude OMP preparation. A primary dose followed by two booster doses was administered until the antibody titers in serum reached 1:64,000 dilutions. The high antibody titer indicated the strong immunogenic nature of the OMP preparation. Serum antibodies from the unimmunized mice showed no reactivity with crude OMP. The polyclonal antibodies from serum reacted with both whole *S. typhimurium* cells by dot ELISA (Fig. 2A) while serum from normal mice did not show any reactivity (Fig. 2B).

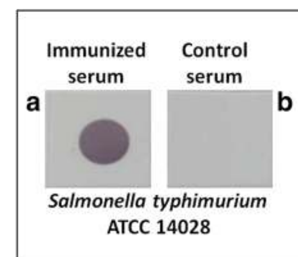

Fig. 2 Dot ELISA performed for testing reactivity of mouse polyclonal anti-*Salmonella* OMP antibodies with *S. typhimurium* whole cells. Panel (**a**) shows reactivity of immune serum with *Salmonella* cells. Panel (**b**) shows no reactivity of unimmunized serum with whole cells

Hybridoma generation

Five monoclonal antibody clones exhibited reactivity with *S. typhimurium* OMP preparation after testing the cell supernatants by indirect ELISA in 96-well microtiter plate. Of the five hybridomas, the reactivity of one mAb named Sal-06 was particularly striking. This clone was expanded further and tested for its reactivity with several whole cells and whole cell lysates of *S. typhimurium* in dot ELISA and western blot analysis respectively. The mAb showed reactivity with all the tested standard and isolated *S. typhimurium* strains in dot ELISA (Fig. 3A) and showed distinct reactivity at ~ 60 kDa region in western blot analysis (Fig. 3B).

Characterization of Sal-06mAb

The properties of Sal-06mAb were characterized by assessing its isotype, reactivity with different *Salmonella* species, and closely related members of *Salmonella*. Isotype analysis revealed that Sal-06mAb clone belongs to IgG1 subclass. Antibody precipitation and concentration procedures did not affect the reactivity of the monoclonal antibody. Sal-06mAb displayed reactivity to all the tested *Salmonella* species such as *S. typhimurium*, *S. typhi*, *S. infantis*, *S. enteritidis*, *S. newportii*, *S. bovismorbificans*, and *S. paratyphi* in western blot analysis (Fig. 4). Due to the conserved nature of epitopes, we are of opinion that the antigen reacting with mAb might be outer membrane protein (OMP) present in all *Salmonella* serovars. The reactivity was seen as distinct protein bands in developed blots at ~ 60 kDa regions. Reactivity of Sal-06mAb was tested with non-*Salmonella* members such as *Escherichia coli*, *Shigella* spp. *Klebsiella pneumoniae*, *Proteus* spp., and *Enterobacter aerogenes*. The mAb was found to react with *E. coli* and *Proteus* spp. (Fig. 5). The bands for these organisms were also found at 60 kDa except for *E. coli* where it was

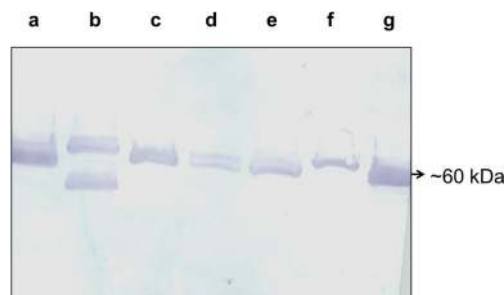

Fig. 4 Reactivity of Sal-06mAb with different species of the genus *Salmonella* tested by western blot analysis. All the tested species showed reactivity with Sal-06mAb indicating a conserved epitope among these members. (a) *S. typhimurium* ATCC 14028. (b) *S. typhi* Gwalior. (c) *S. infantis* MTCC 1167. (d) *S. enteritidis* isolate Gwalior. (e) *S. newporti isolate*. (f) *S. bovismorbificans* MTCC 1162. (g) *S. paratyphi* MTCC 735

found to be less than 60 kDa. By western blot patterns, we observed that *Salmonella* shares some common antigens on their outer membrane with other members of *Enterobacteriaceae*.

Anti-microbial studies of Sal-06mAb

Since the Sal-06mAb was found to be reactive with OMP of different *Salmonella* serovars, we investigated if the mAb possess bacteriostatic/ bactericidal properties. We utilized the standard *S. typhimurium* ATCC 14028 strain for testing the antimicrobial activity. A standard dilution of 10^4 CFU/well in MH broth with different dilutions of mAbs was incubated for 18 h. We found that mAb was able to control the growth of the bacteria temporarily. There was visible growth in all the wells; however, differences in the optical density were very clear with varying concentrations of the mAb. Figure 6 depicts a clear distinction of growth curves of the bacteria under various concentrations of mAb. At the highest concentration of mAb,

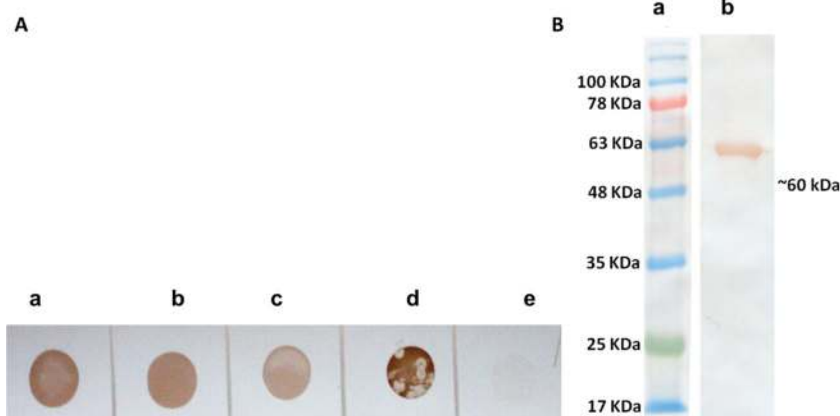

Fig. 3 Reactivity testing of Sal-06mAb by dot ELISA. Dot ELISA was performed with whole cells of different members of the genus *Salmonella*. Panel **A**: (a) *S. typhimurium* ATCC 14028. (b) *Salmonella typhi* Gwalior isolate. (c) *Salmonella paratyphi* MTCC 735. (d) Crude OMP extract. (e) Blank. Western blot analysis. Western blot was performed with whole cell lysate of *S. typhimurium* ATCC 14028 strain. Panel **B:** (a) Premix protein marker. (b) *Salmonella* OMP extract 1

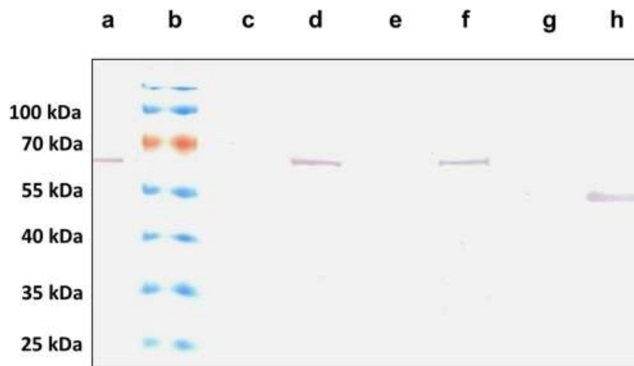

Fig. 5 Reactivity of Sal-06mAb was tested with different members of the family Enterobacteriaceae by western blot analysis. *Proteus mirabilis* and *E. coli* showed reactivity with Sal-06mAb indicating a conserved epitope among these members. (a) *S. typhimurium* ATCC 14028. (b) Prestained protein marker. (c) *Shigella flexneri* NCIM 5265. (d) *S. typhimurium* NCIM 5278. (e) *Klebsiella pneumoniae* ATCC 13883. (f) *Proteus mirabilis* MTCC 3310. (g) *Enterobacter aerogenes* ATCC 13048. (h) *E. coli* ATCC 10536

the growth of the bacteria has reduced considerably. In other words, the growth is inhibited but not completely stopped. The growth has resumed after ~6 h of inoculation, where as in control wells with bacteria and no mAb, the growth resumed immediately. As we decreased the mAb concentration, the time lapse between the control wells and mAb-treated wells was decreasing. To confirm the specificity of inhibitory effect Sal-06mAb to *Salmonella*, *Salmonella* cells were treated with 120 μg of a control mAb (mouse monoclonal anti-histidine antibody—Sigma Aldrich) which did not affect the growth of bacteria from that of untreated control. Therefore, we concluded that the inhibitory effect of mAb was time and concentration dependent. Also, the growth of the bacteria was inhibited for 4–6 h but not indefinitely indicating that the effect is bacteriostatic.

Effect on complement on antimicrobial activity

Complement is part of innate immune system but can be brought in to work by adaptive (humoral) immune system. Complement enhances the activity of antibodies and phagocytic cells to clear pathogens, promotes inflammation, and attacks the pathogens' cell membrane. Therefore, we investigated whether the bacteriostatic effect of mAb was complement dependent. We collected the mouse serum containing the complement and used directly. We conducted the growth curve assay as mentioned above along with complement added to each well. Figure 7 shows the effects of mouse complement system on bacteriostatic effects of mAb. It was revealed that there was no improvement in the reduction of growth in the wells containing complement.

Invasion inhibition assay

Adherence to intestinal epithelial cells is the first step in invasion process of the intestinal pathogen. We investigated whether the mAb in the present study would inhibit the pathogen from invading the host cells. One batch of cell lines (epithelial cell line A549) was grown in tissue culture plates for CFU count analysis and another batch was grown on cover slips for microscopy. After incubation with cell lines with and without mAb, the adherent bacterial cells were washed with antibiotics. The bacterial cells invaded into the host cell lines were enumerated after lysis. Simultaneously, the invasion was observed by fluorescent microscope. We found that the bacterial cells were found to invade in the absence of mAbs. The presence of mAb inhibited *Salmonella* invasion which can be observed in Table 1 that the bacterial cells preincubated with mAbs at bacteriostatic concentrations failed to invade the cells; hence, there were no colonies observed after the cell lines were lysed and plated on plate count agar (Table 1).

We also observed the invasion of bacterial cells under phase contrast and fluorescent microscope. We found that the cells incubated with pathogen alone were having damaged cell membranes (Fig. 8B, D). It was observed that the A549

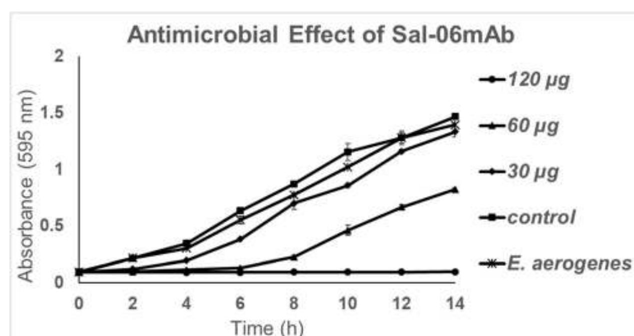

Fig. 6 Bacteriostatic/bactericidal potential of Sal-06mAb was tested by microtiter-based anti-microbial assay. The Sal-06mAb exhibited bacteriostatic property at 120 μg concentration. The antibacterial activity is both concentration and time dependent

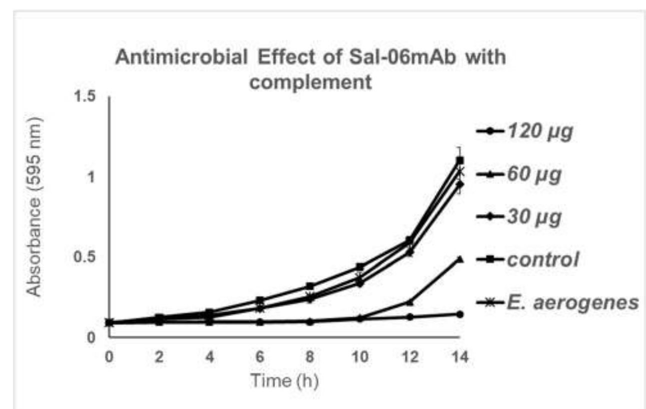

Fig. 7 Bacteriostatic/bactericidal potential of Sal-06mAb was tested in the presence of mouse complement by microtiter-based anti-microbial assay. The presence of serum complement did not improve the bacteriostatic property

Table 1 Colony count of invasion inhibition assay. Number of bacteria invaded A549 cell lines when treated with Sal-06mAb

Sal-06mAb concentration	Number of colonies (CFU)
120 μg	0
60 μg	0
30 μg	28
Control	1.2×10^3

cells had leaked their cytoplasmic content due to the cellular damage made by the pathogenic bacteria. In contrast, the cell lines were intact when they were invaded by the pathogen preincubated with the mAb (Fig. 8A, C).

SEM analysis

The mAb-treated *Salmonella* exhibited distinct morphological alterations in comparison with untreated bacterial cells. Control cells were intact, homogenous, and turgid with smooth membrane (Fig. 9A). Monoclonal antibody-treated *Salmonella* cells showed severe morphological alterations such as flaccid/squashed cells with uneven wrinkles, protuberances and craters, and collapsed membrane with disrupted cell contents (Fig. 9B). Addition of mouse complement did not favor these morphological damages at bacteriostatic concentrations of monoclonal antibody. Increasing the incubation time promoted damage of cell membranes by mAb (Fig. 9C). From this, we inferred that Sal-06mAb was able to bind outer membrane proteins and was disrupting the integrity of cell membrane.

Discussion

Globally, *Salmonella* is the predominant cause of typhoidal and non-typhoidal gastroenteritis infections leading to significant mortality and morbidity (Liu et al. 2018). Non-typhoidal *Salmonella* alone causes ~ 93 million cases of gastroenteritis worldwide annually (Eng et al. 2015). Emergence of multi-drug-resistant strains has limited the antibiotic treatment options. Vaccines present an attractive approach to control and prevent *Salmonella*-associated diseases. Several of the reported vaccines are only experimental or are presently under development. Reports from animal and human studies support the possibility of a safe and effective vaccine against invasive non-typhoidal *Salmonella*. Studies from animal models demonstrated the efficacy with O-antigens, flagellin proteins, and

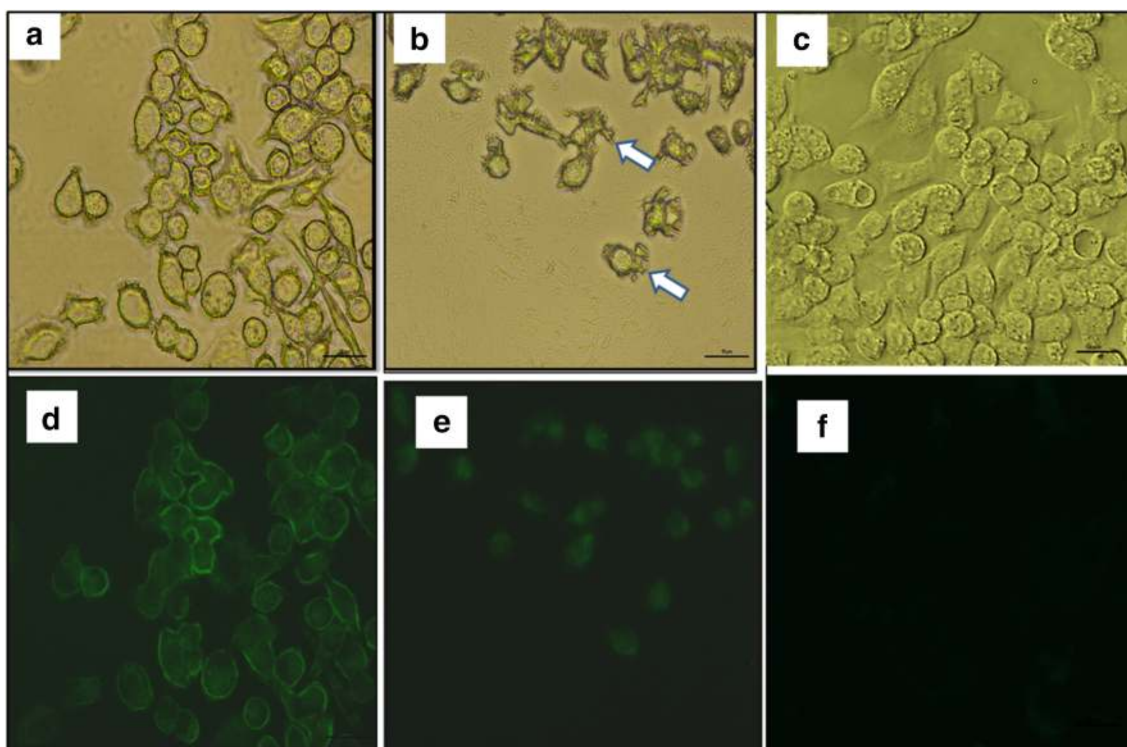

Fig. 8 Study of invasion inhibition ability of Sal-06mAb in vitro on A549 epithelial cell line by phase contrast and fluorescent imaging. Panel **a**) Phase contrast image of epithelial cells incubated with *Salmonella* bacteria and Sal-06mAb. **b**) Phase contrast imaging of epithelial cells incubated with *Salmonella* bacteria alone (arrow mark shows the damaged cells). **c**) Phase contrast imaging of epithelial cells alone. **d**) Fluorescent imaging of epithelial cells incubated with *Salmonella* bacteria and Sal-06mAb. **e**) Fluorescence imaging of epithelial cells incubated with *Salmonella* bacteria alone. **f**) Fluorescence imaging of epithelial cells alone

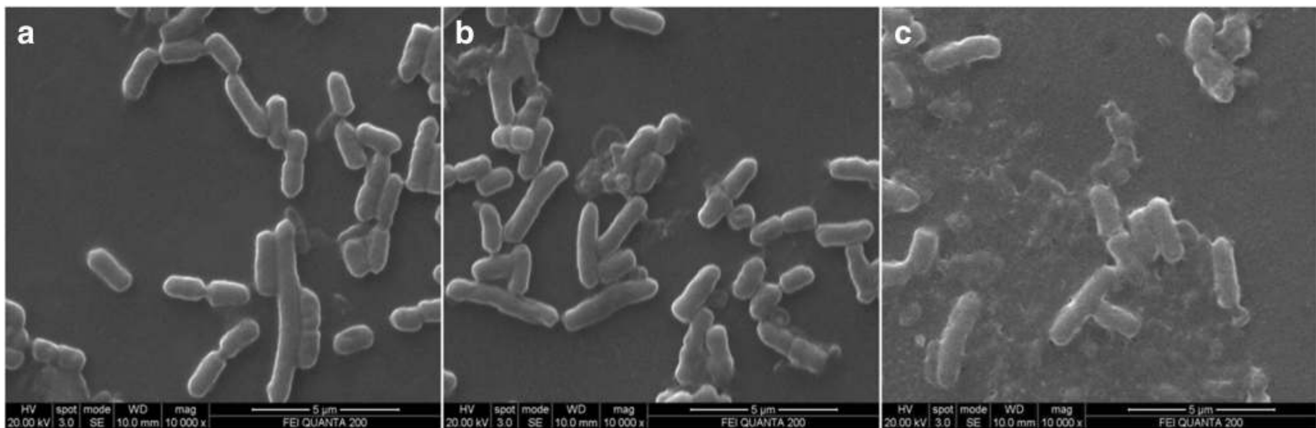

Fig. 9 Study of the effect of Sal-06mAb on morphology of *S. typhimurium* by a scanning electron microscope. **a**) *Salmonella* bacteria without mAb incubation (control). **b**) *Salmonella* cells incubated with Sal-06mAb for 1 h. **c**) *Salmonella* cells incubated with Sal-06mAb for 2 h

outer membrane proteins of *typhimurium* and *enteritidis* serovars (Tennant et al. 2016). Though the live attenuated vaccines are able to confer cross protection, the risk of virulence restoration in immune-compromised individuals is limiting their use (Haesebrouck et al. 2004). This led to focus on developing novel and alternative antimicrobial therapies offering high specificity, improved safety, less toxicity, broad immunity, and cross-protective efficacy against broad *Salmonella* serovars. Passive immunization has been utilized successfully as a suitable alternative to conventional antibiotic therapy against several gastrointestinal pathogens such as *Salmonella*, *Campylobacter*, *Escherichia coli*, and rotavirus (Esmailnejad et al. 2019; Rahimi et al. 2007). Antibodies exert their antimicrobial activity through binding and blocking the pathogen entry, neutralization, inhibiting growth, replication and dissemination, and membrane damage (Peddayelachagiri et al. 2014; Kovacs-Nolan and Mine 2004; Esmailnejad et al. 2019). Monoclonal antibodies have found their way in immunodiagnostics, pathogen identification, prevention, and therapeutic applications. Administration of monoclonal antibodies raised against conserved antigens of *Enterobacteriaceae* members might confer a neutralizing effect by antibody-dependent cellular cytotoxicity (ADCC) effect.

Several earlier studies have explored the possibility of immunotherapy utilizing cross-reactive monoclonal antibodies against broad range of *Enterobacteriaceae* pathogens. A murine monoclonal antibody (WN1 222-5) capable of binding to LPS of *E. coli* and *Salmonella* has shown in vitro and in vivo cross-protective activity against LPS-induced lethality in mouse model (Di Padova et al., 1993). In another study, two monoclonal antibodies ST-1 and 10-5-6 directed against the O-polysaccharide of *S. typhimurium* LPS provided significant protection against the lethal challenge with homologous *S. typhimurium* strains (SR-11 and LT-2) in the C3H/HeN mice (Colwell et al., 1984). Michetti et al. (1992) reported the protective efficacy of a secretory IgA challenge by an invasive *S. typhimurium* by immune exclusion at the mucosal surface. In a similar study, permanent secretion of mucosal IgA (mAb 177E6) directed specifically against O:9 antigen into the respiratory tract through a backpack tumor model protected 50% of animals challenged with high dose of *S. enteritidis* strain through intranasal route (Iankov et al., 2004). Our work is in agreement with the above studies wherein a cross-reactive, surface protein targeted monoclonal antibody can neutralize the pathogen, thereby blocking its entry into the cells. Sal-06 monoclonal antibody was reactive to several species of *Salmonella* on dot ELISA and western blot analysis. Since several members of the family Enterobacteriaceae are closely related in structure, composition, and virulence mechanisms, we tested the cross-reactivity of Sal-06 monoclonal antibody on other members of genus such as *Klebsiella*, *Proteus*, *Escherichia*, and *Enterobacter*. The monoclonal body showed strong cross-reactivity with *K. pneumonia*, *P. vulgaris*, and *E. coli* indicating shared epitopes among these organisms. These cross-reactive pathogens are also of great clinical significance. Western blot analysis revealed the size similarity of cross-reactive antigen among the tested pathogens with that of *Salmonella* members. Antigen reactive to the Sal-06mAb was assumed to be of proteinaceous in nature and not a lipopolysaccharide (LPS) based on the distinct banding pattern of reactivity as opposed to ladder-like formation usually associated with LPS (Peddayelachagiri et al. 2014). Antibodies directed against bacterial surface components might prevent pathogen from establishing infection by blocking their binding sites, adhesion, and invasion into host cells. This effect is reflected in vitro on the rate of growth and survival of the bacteria. We observed that under in vitro conditions, the Sal-06mAb inhibited the growth of actively dividing *Salmonella* bacteria for more than 4 h at 60 μg quantity. We assume that such effect under in vivo conditions would be sufficient to induce opsonization and pathogen neutralization. This hypothesis needs to be studied in vivo in suitable animal model. In Sal-06mAb, the mAbs bound to the pathogen surface and blocked their invasion into the A549 epithelial cell line. Adhesion of

bacteria to the cell surface is the primary step in the establishment of infection. Prevention of bacterial invasion is promising in prevention of establishment of pathogenesis (Peddayelachagiri et al. 2014). Further, Sal-06 being an IgG1 subclass of mAb might display potent neutralization and phagocytic killing ability in vivo. SEM studies further complemented our assumptions of the surface location of antigen specific to Sal-06. In electron micrographs, it was clearly evident that mAb bound to surface epitopes and induced osmotic lysis of the bacterial cells which demonstrate its partial bacteriostatic action.

In conclusion, our work resulted in development of Sal-06mAb broadly reactive to few members of the family *Enterobacteriaceae* and demonstrated bacteriostatic properties against *Salmonella* species under in vitro conditions. The Sal-06mAb demonstrated broadly cross-reactive, bacteriostatic, and invasion inhibitory properties. Due to the shared epitopes among many pathogenic members of *Enterobacteriaceae*, the Sal-06mAb could be explored as a valuable therapeutic tool. However, additional and thorough in vivo preclinical and clinical validations need to be performed to realize this antibody for therapy. Approaches such as recombinant single-chain variable fragment (SCFV) antibodies and humanization strategies may also be explored. Further, the identity of antigen and epitope binding to Sal-06mAb also needs to be identified.

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Compliance with ethical standards

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All the animal experiments were conducted as per Institutional Animal Ethics Committee guidelines.

Conflict of interest The authors declare that they have no conflict of interest.

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