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Recombinant Flagellin and Incomplete Freund's Adjuvant Potentiate the Vaccine Efficacy of the Iron Acquisition Protein (HitA) of *Pseudomonas aeruginosa*

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ABSTRACT

Objectives*: Pseudomonas aeruginosa* is a notorious bacterial pathogen that can cause a variety of infections with high morbidity and mortality. The increasing multi-drug resistance of this pathogen makes it urgent to develop an effective vaccine. This study aimed at evaluation of the immunoadjuvant effect of flagellin (FliC) of *Salmonella enterica* and incomplete Freund's adjuvant (IFA) on the protective vaccine efficacy of *P. aeruginosa* recombinant iron acquisition protein (HitA). **Methods**: In this work, recombinant HitA, FliC and fused FliC-F-HitA proteins were expressed in *Escherichia coli* after cloning their respective genes into pQE31 plasmid vector. The proteins were purified using metal affinity chromatography. The immunoadjuvant effect of FliC was examined upon mixing as well as fusion with HitA antigen of *P. aeruginosa* in the presence of incomplete Freund's adjuvant (IFA). This was tested by active immunization followed by challenge using *P. aeruginosa* murine infection model. **Results**: Two weeks after the last immunization dose, serum samples were tested for antibody response which showed significant HitA-specific IgG antibody response in all immunized groups compared to control groups. A significant reduction in bacterial burden of lungs from mice immunized with HitA/FliC/IFA mixture was observed after challenge. Opsonophagocytic assay and liver histopathological examination confirmed the previous results. **Conclusion:** Overall, HitA recombinant protein is considered a promising vaccine candidate against *P. aeruginosa* upon mixing with *S. enterica* flagellin protein FliC and IFA.

Keywords*: Pseudomonas aeruginosa; Vaccine; HitA; Flagellin; FliC; Iron acquisition protein*

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic, ubiquitous, Gram negative rod-shaped bacterium. It can cause serious infections especially in immunocompromised patients such as cancer, burn, diabetic and cystic fibrosis patients[1]. It can cause respiratory tract infection, urinary tract infections (UTIs), bacteremia, and soft tissue infections[1]. Treatment of *P. aeruginosa* infected patients constitutes a difficult challenge due to high intrinsic resistance to antibiotics because of numerous multiple drug efflux

pumps and low permeability of the outer membrane of the pathogen[2]. It also has the capability of forming biofilms on surfaces of medical devices such as catheters, ventilators, in addition to mucosal membranes[3]. Up till now, no licensed vaccine is available against *P. aeruginosa* infection despite the tremendous efforts made during the last four decades[4]. Different types of vaccines were developed using different cell associated and secreted *P. aeruginosa* antigens such as the mucoid substance, high molecular mass alginate components, surface exposed antigens (O and H antigens), polysaccharides, polysaccharide-protein conjugates, outer membrane proteins F and I, the type III secretion system component PcrV, pili and live attenuated vaccines[5–7]. The challenge of developing a protective vaccine against this pathogen is due to many factors such as high serotype variability of immunogenic epitopes and low immunogenicity of conserved epitopes[6], in addition to the high variability of host immune response due to different health conditions of people at risk to *P. aeruginosa* infection[7]. Using immunoadjuvants with vaccines, especially subunit ones, is very important to elicit strong immune response following immunization[8]. Flagellin of *Salmonella enterica* serovar Typhimurium is a potent Toll-like receptor-5 (TLR-5) agonist, and its immunostimulatory activity is well-reported[9]. Incomplete Freund's adjuvant (IFA) is another important immunoadjuvant that is composed of mineral oil and a surfactant called mannide monooleate[10]. IFA is mixed with aqueous solution of antigen to form a water-in-oil emulsion. It is commonly used as immunoadjuvant in animal experiments to elicit strong antibody response against co-administered vaccine antigens[10].

Many virulence factors contribute to *P. aeruginosa* pathogenicity, but the iron acquisition systems plays an important role in the virulence of this pathogen especially in chronic infections[11–13]. This creates an interesting field of research for the potential use of iron acquisition proteins as vaccine candidates against *P. aeruginosa* infection.

The iron acquisition periplasmic protein HitA plays an important role in iron acquisition by *P. aeruginosa* and other Gram-negative bacteria[14,15]. We recently reported on the potential of HitA as a *P. aeruginosa* vaccine candidate in combination with Bacillus Calmette-Guérin (BCG) as an immunoadjuvant[16]. We herein elaborate on that work by testing further immunoadjuvants, namely recombinant flagellin and incomplete Freund's adjuvant (IFA).

MATERIAL AND METHODS

Microorganisms

P. aeruginosa standard strain (ATCC 9027) and *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC 14028) were used for PCR amplification of *hitA* gene and *fliC* gene, respectively. *E. coli* DH5α and *E. coli* M15 (pREP4) were used as intermediate cloning and expression hosts, respectively.

Culture media, antibiotics and incubation conditions

Luria Bertani (LB) broth (10 g/l Tryptone (Oxoid, USA), 5 g/l Yeast extract (Oxoid, USA), and 10 g/l Sodium chloride (MP Biomedicals, France)) was used for inoculation of all bacteria used in this study. LB agar was prepared by adding agar agar (2% w/v) to prepared LB broth before autoclaving. Ampicillin (Epico, Egypt) and kanamycin sulfate (Sigma Aldrich, USA) were added to the culture media at final concentration of 100µg/mL and 25µg/mL whenever required. All liquid cultures used in this study were incubated for 18h at 37°C and 200 rpm except for cultures of induction for protein expression which were incubated for 3h at 37°C and 200rpm.

In silico analysis of HitA immunogenicity

The immunogenicity of HitA antigen (protein accession NP_253376.1) was analyzed using two online antigen prediction tools, namely, VaxiJen (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) and AntigenPro program from SCRATCH tools (http://scratch.proteomics.ics.uci.edu/).

PCR amplification and splicing by overlap extension of *hitA* and *fliC* genes:

The DNA sequence of *hitA* gene and *fliC* gene (Accession no. NC_002516.2 and KF589316.1) were checked for the presence of signal peptide sequence using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP-4.1/). Oligonucleotide primers were designed to exclude the coding sequence of HitA signal peptide during polymerase chain reaction (PCR) amplification. **Table 1** summarizes the oligonucleotide primers used in this study. *P. aeruginosa* and *S. enterica* colonies, isolated on LB agar plates, were used to amplify *hitA* (927 bp) and *fliC* (1488bp) genes by colony PCR, respectively. For *hitA* amplification, primers 1 and 2 were used while primers 3 and 4 were used for *fliC* amplification. Fusion of *fliC* and *hitA* genes was carried out using the splicing by overlap extension (SOE) technique[17]. Briefly, *fliC* was amplified with primers 3 and 5 while *hitA* was amplified with primers 6 and 2. The two amplicons were gel extracted after agarose gel electrophoresis then mixed at equal molar ratio to act as a template for a third PCR reaction using primers 3 and 2 to result in the fusion amplicon *fliC-F-hitA*. **Figure 1** shows a diagrammatic illustration of the PCR amplification procedures of target amplicons. MyTaq™ hot start

Table 1. Oligonucleotide primers used in the current study

Primer code	Primer name	Primer sequence (5' to 3') [a]
1	*hitA* forward primer	ATTCGGATCCGGATCCCGTCACCCTTACCCT (BamHI)
2	*hitA* reverse primer	ATTCAAGCTTTCAATTCAGGCCAACGTCGC (HindIII)
3	*fliC* forward primer	ATCCGGATCCGATGGCACAAGTCATTAATAC (BamHI)
4	*fliC* reverse primer	ATCGAAGCTTTTAACGCAGTAAAGAGAGGA (HindIII)
5	*fliC* reverse primer with *hitA* overhang	GGGTGACGGGATCACGCAGTAAAGAGAGGACGT
6	*hitA* forward primer with *fliC* overhang	TCTCTTTACTGCGTGATCCCGTCACCCTTACCCT

[a] *When applicable, recognition sites of restriction enzymes are underlined and enzyme name is mentioned between parentheses. For primers 5 and 6, overhangs are underlined.*

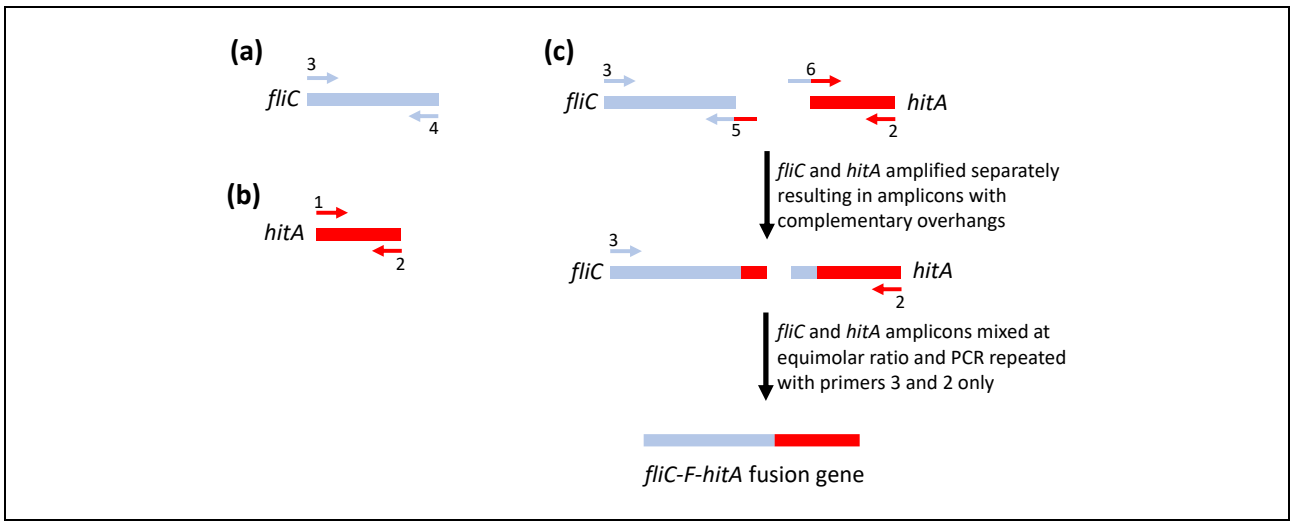

Figure 1. Simplified illustration of PCR amplification of target amplicons. (a) and (b) represent amplification of *fliC* and *hitA*, *respectively*, using corresponding gene-specific primers. (c) shows the steps of splicing by overlap extension (SOE) technique to construct the fusion gene *fliC-F-hitA*. Primer numbers (according to Table 1) are mentioned adjacent to the arrows representing each pertinent used primer. The three amplicons resulting from (a), (b) and (c) were digested with BamHI and HindIII and ligated to a similarly digested pQE31 plasmid for cloning and expression of the corresponding protein products.

Master Mix (BIOLINE, UK) was used in all PCR reactions following manufacturer's instructions. Plasmid pQE31 as well as all amplicons were digested with BamHI and HindIII. Ligation of each amplicon to pQE31 was fulfilled individually using T4 DNA ligase (NEB, USA). Chemically competent *E. coli* DH5α cells were transformed with the ligation reactions and positive clones were selected by inoculation on ampicillin plates. One transformed colony with each constructed plasmid was selected and grown for plasmid extraction. Chemically competent *E. coli* M15 (pREP4) was transformed with the created plasmids and positive clones were selected on ampicillin/kanamycin LB agar plates. The constructed plasmids, pQE31-*hitA*, pQE31-*fliC* and pQE31-*fliC-F-hitA*, were extracted and sent for insert sequencing by GATC Biotech sequencing services (Germany).

Protein Expression of recombinant HitA, FliC and fusion FliC-F-HitA

Induction of protein expression was carried out as outlined in the QiaExpressionist™ manual of Qiagen (Germany). A fresh overnight culture of *E. coli* (pREP4) harboring one of the constructed plasmids was

used to inoculate 200mL LB broth supplemented with ampicillin and kanamycin. The culture was incubated at 37°C with vigorous shaking till reaching optical density at 600nm (OD600) of 0.4-0.6. This was followed by induction with isopropyl β-D-1-thiogalactopyranoside (Melford, UK) at a final concentration of 1mM. After induction of recombinant protein expression, cells were harvested by centrifugation, washed with sterile 0.9% saline and the bacterial pellet was kept at -20°C until protein purification was carried out.

For protein purification, the frozen bacterial pellet was thawed on ice for 15min and re-suspended in 10 mL of denaturing lysis buffer (100mM NaH_2PO_4, 10mM Tris.Cl and 8M Urea in distilled water; pH 8) and left for lysis at 25°C for 1h at 80rpm horizontal shaking. After bacterial lysis, cell lysate was collected after centrifugation at 4°C for 30min at 12000 xg and recombinant His-tagged antigens were purified using Nickel-NitriloTriacetic Acid (Ni-NTA) agarose (Qiagen, Germany) under denaturing conditions (The QiaExpressionist™, Qiagen, Germany). Purified recombinant antigens were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Acrylamide/Bis-acrylamide 40% (37.5:1) (Noragen Biotek corp., Canada) in the preparation of separating gel (12%) and stacking gel (4%). SDS-PAGE gel was stained using Coomassie brilliant blue R-250 dye (MP Biomedicals, USA) and destained using a solution of 200mL glacial acetic acid, 100mL methanol and 700mL distilled water. To confirm that purified antigens are His-tagged proteins, western blot assay was performed as previously described[18] using mouse anti-His tag antibody (Biolegend, USA) as the primary antibody. The secondary antibodies used were alkaline phosphatase-labeled antibody to mouse IgG (KPL, USA) in case of HitA antigen and peroxidase-labeled antibody to mouse IgG in case of FliC and FliC-F-HitA. Detection was performed using BCIP/NBT phosphatase substrate (1-component) (KPL, USA) to detect HitA and 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (Bio Basic Inc., Canada) to detect FliC and FliC-F-HitA. In addition, western blot against HitA protein was also carried out using mouse anti-*Pseudomonas aeruginosa* serum (obtained from mice immunized with sublethal *P. aeruginosa* infection) as the primary antibody in order to check HitA immunogenicity. Recombinant protein eluates were refolded using PD-10 desalting columns (GE Healthcare, USA) containing Sephadex™ G-25 medium following manufacturer instructions.

Mice immunization with recombinant antigens

Eight-week-old female Swiss albino mice (20-25g) were purchased from Theodor Bilharz Research Institute, Giza, Egypt. The mice were divided into groups (8 mice/group). All animal procedures were approved by the Animal Care and Use Committee (ACUC) of the Faculty of Pharmacy, Alexandria University. All animal experiments were performed following institutional and international ethical standards. In this study, two adjuvants were used, recombinant FliC antigen and incomplete Freund's adjuvant (IFA). Five groups of mice were used to assess the protective effect of HitA recombinant protein against *P. aeruginosa* infection using different immunization regimens. Group 1 mice were immunized with a mixture of HitA (10µg), FliC (6µg) and IFA, group 2 mice were immunized with the fusion protein FliC-F-HitA (10µg) and IFA, group 3 mice were immunized with HitA (10µg) and IFA, group 4 mice were injected with IFA in saline and group 5 were immunized with HitA (10µg) only. Each group was immunized by subcutaneous injection with 200µL of prepared regimen followed by two booster doses at two-week intervals. Two weeks after the last booster dose, blood samples were collected from mice by the submandibular bleeding method[19], and sera were kept at -20°C for subsequent analysis.

Indirect enzyme linked immunosorbent assay (ELISA)

Refolded HitA recombinant antigen was diluted to a final concentration of 10µg/mL with coating buffer (2.65g sodium carbonate and 2.1g sodium bicarbonate per 500mL sterile distilled water, pH 9.6). Diluted antigen was used for coating 96-well high binding ELISA plate (Greiner Bio One, Germany) (100µl/well). Coated plate was incubated statically overnight at 4°C. After coating, the plate was washed 3 times with phosphate-buffered saline (PBS) followed by blocking with 5% skimmed milk 3h. After blocking, the plate was washed 3 times with PBS and 100µl of diluted mouse serum (1:300 dilution in blocking buffer) were added and left for 1h. After thorough washing, peroxidase-labeled anti-mouse IgG antibody (KPL, USA) was added for 1h. After washing the plate, 100µL of 3,3',5,5'-Tetramethylbenzidine (TMB) Microwell Peroxidase Substrate System (KPL, USA) were added to each well and left for color development in darkness for 20min at room temperature. This was followed by adding 50µL of 1M sulfuric acid (stopping solution) and measuring the absorbance at 450nm using microplate reader (BIOTEK, USA).

Opsonophagocytic assay

Serum samples were collected 13 days after the last vaccine booster before the bacterial challenge. The opsonophagocytosis stimulating activity of serum samples isolated from immunized mice were examined as previously described[16,20]. Briefly, *P. aeruginosa* was inoculated into RPMI 1640 medium containing 10% v/v fetal bovine serum (FBS) and incubated at 37°C for

18h. Grown bacterial cells were harvested by centrifugation at 4°C followed by washing with sterile saline and final resuspension in 2mL sterile saline. Human peripheral polymorphonuclear leukocytes (PMN) were freshly isolated from blood of a healthy human volunteer using Histopaque®-1077 (Sigma Aldrich, USA) according to manufacturer's instructions and following informed consent by the blood donor. The isolated PMNs were resuspended into 500µL of RPMI 1640 medium, and cell viability was determined by trypan blue exclusion method. PMN cells were diluted to a final count of $5X10^5$ cells/ml using the same medium. Reaction mixture was prepared by mixing 5µL of diluted bacterial suspension (1500 colony forming units (CFU)), 20µL pooled mouse serum (heat inactivated at 56°C for 10min), 15µL PMNs (7500 cells) and 20µL active complement (fresh human serum). Samples were taken at zero time and 40 minutes later following shaking incubation at 37°C. Bacterial count was determined by plating tenfold serial dilutions onto LB agar plates where the grown colonies after incubation at 37°C for 18h were counted and original count was calculated. Result was calculated as percentage killing using the formula: [(count of control – count of immunized)/count of control] X100.

Mice challenge with *P. aeruginosa*

Mice in this study were challenged two weeks after the last immunization dose by intraperitoneal injection of 200µL bacterial suspension equivalent to 10^8 CFU. Inoculum of *P. aeruginosa* was prepared from overnight culture in RPMI 1640 medium containing 10% FBS. Mice were sacrificed 48h after the challenge for organ examination. Lungs were isolated for bacterial bioburden determination. Isolated lungs were homogenized in 3mL sterile saline, tenfold serially diluted and then plated on LB agar plates. Livers were also isolated and fixed in 10% formalin and examined microscopically after hematoxylin and eosin (H&E) staining for histopathological changes due to *P. aeruginosa* infection. Parameters examined were focal lytic necrosis, bile ductular proliferation, congestion, Kupffer cells hyperplasia, steatosis and ballooning degeneration of hepatocytes and periportal inflammation[21,22]. Scoring systems were used for quantitative comparison between immunized and non-immunized mice[23,24].

Statistical analysis

ELISA results of IgG antibody response in different mice groups were analyzed using one-way analysis of variance (ANOVA) followed by post-test (Tukey-Kramer test). Lung bioburden and liver histopathological scores were analyzed using Kruskal Wallis non-parametric test and Dunn's post-hoc test. Statistical analysis tests were performed using GraphPad InStat 3. P value was considered significant when less than 0.05.

RESULTS

Immunogenicity prediction, PCR amplification and splicing by overlap extension

Bioinformatic analysis of HitA protein by VaxiJen and AntigenPro antigen prediction tools revealed antigenic score of 0.48 (above the threshold of 0.4) and antigenic probability of 0.788, respectively. Amplification of *hitA* and *fliC* with gene-specific primers was carried out by colony PCR on *P. aeruginosa* and *S. enterica* isolated colonies, respectively. SOE technique was successfully employed to create the fusion gene *fliC-F-hitA*. Agarose gel electrophoresis revealed the amplicons at their expected sizes: *hitA* gene (927bp), *fliC* gene (1488bp) and the fusion gene *fliC-F-hitA* (2415bp) (**Figure 2**). *E. coli* (pREP4) clones harboring the different constructed plasmids were confirmed by gene-specific PCR reactions and DNA sequencing.

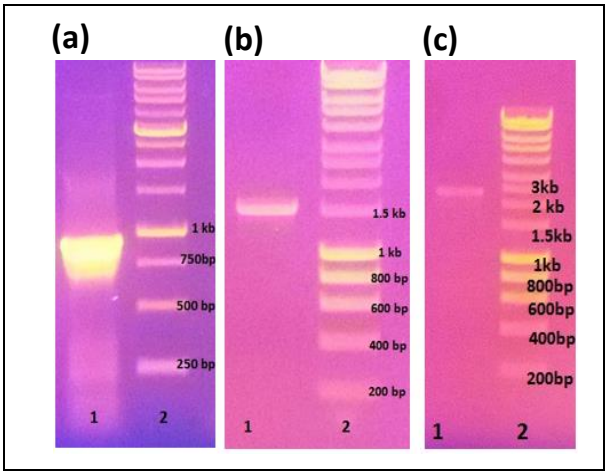

Figure 2. Agarose gel electrophoresis of gene amplicons (a) PCR amplified hitA (927bp) (Lane 1) using gene-specific primers and *P. aeruginosa* colony PCR. (b) PCR amplified fliC (1488bp) (Lane 1) using gene-specific primers and an isolated S. enterica colony PCR. (c) PCR amplified fusion gene fliC-F-hitA using primers 3 and 2 showing a band at the expected size of 2415bp (Lane 1). Lane 2 represents DNA ladder.

Successful antigen expression in *E. coli* (pREP4) hosts

Recombinant expressed antigens were purified using Ni-NTA agarose to capture His-tagged antigens followed by washing and elution of pure antigens. Purified antigens were checked by SDS-PAGE as shown in **Figure 3**. All the three recombinant antigens;

HitA, FliC and FliC-F-HitA, appeared at their expected sizes: 34, 53 and 88 kDa, respectively. Recombinant antigens identity, as being His-tagged, was confirmed by western blot analysis using mouse anti-His tag antibodies (**Figure 4**). Furthermore, mouse anti-*Pseudomonas aeruginosa* serum antibodies reacted positively with HitA in western blot confirming immunogenicity of HitA upon *P. aeruginosa* infection (data not shown).

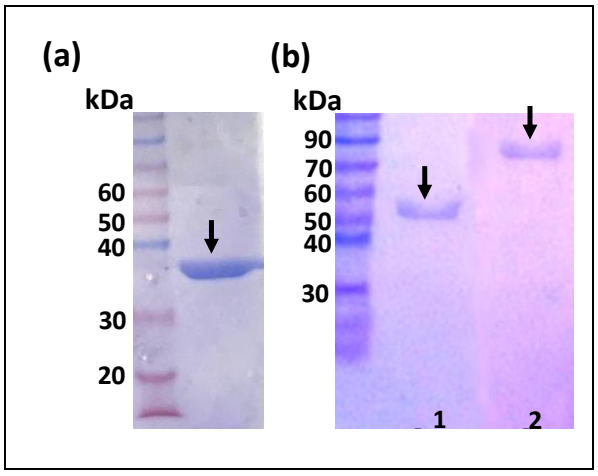

Figure 3. SDS-PAGE of the recombinant expressed antigens. (a) SDS-PAGE of purified HitA antigen (34 kDa). (b) SDS-PAGE of purified FliC (53 kDa) (lane 1) and the fusion FliC-F-HitA protein (about 88 kDa) (lane 2).

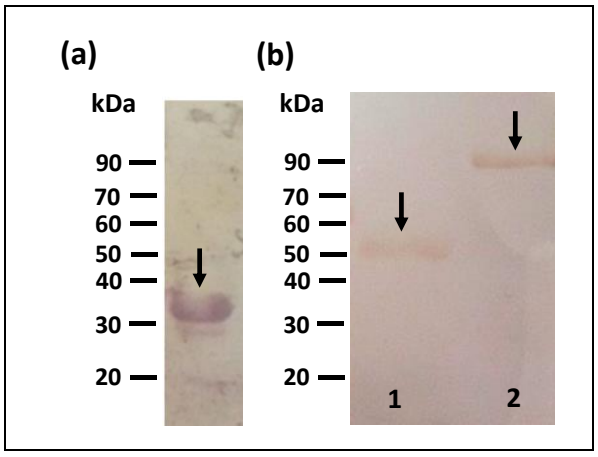

Figure 4. Western blot to detect His-tagged antigens. (a) Western blot of HitA using mouse anti-His tag antibody as primary antibody and detection by BCIP/NBT phosphatase chromogenic substrate. **(b)** Western blot of FliC (lane 1) and the fusion FliC-F-HitA protein (lane 2) using mouse anti-His tag antibody as primary antibody and detection by 3, 3'-diaminobenzidine tetrahydrochloride (DAB) peroxidase chromogenic substrate.

Active immunization elicits HitA-specific IgG antibodies which enhance *P. aeruginosa* opsonophagocytosis by PMNs

Indirect ELISA results showed a significant HitA-specific IgG antibody response in immunized groups 1, 2 and 3. On the other hand, negligible IgG response was observed in the IFA negative control group 4, and in group 5 immunized with HitA alone without adjuvants (**Figure 5)**. Opsonophagocytic assay demonstrated that serum antibodies from immunized groups 1, 2 and 3 enhanced the phagocytic activity of PMNs. No *P. aeruginosa* count was recovered from opsonophagocytic reactions containing serum from immunized mice reflecting 100% percentage killing compared to the negative control group and the group given HitA alone.

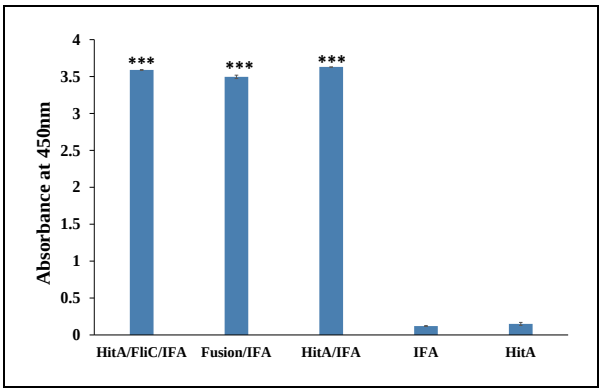

Figure 5. Indirect ELISA results showing HitA-specific immune response. Murine HitA-specific IgG antibody was measured two-weeks after the last vaccine booster. The illustrated results are at serum dilution of 1/300. Error bars represent mean reading+/- SEM (standard error of the mean).

Immunized mice are protected against *P. aeruginosa* challenge

Animal groups were challenged with *P. aeruginosa* intraperitoneally two weeks after the last vaccine booster. After 48h, mice were sacrificed to determine bacterial count in lungs. There was a significant reduction in bacterial burden of lungs in group 1 compared to group 4 and 5 ($P < 0.01$) as shown in **Figure 6**. There was no statistically significant lung protection in other immunized groups.

Upon examining the liver sections of the immunized groups, they showed preserved lobular architecture with no cirrhotic changes or fibrosis, no/minimal focal lytic necrosis, low portal inflammation, no bile ductular proliferative inflammation, minimal central or portal veins congestion and no Kupffer cells hyperplasia (**Table 2** and **Figure 7**). There was a significant reduction in

Table 2. Histopathological scoring of livers isolated from immunized and non-immunized mice

Group number	Ballooning degeneration of hepatocytes (0-2)	Bile ductular proliferation (0-3)	Congestion (0-3)	Focal lytic necrosis (0-4)	Kupffer cells hyperplasia (0-3)	Periportal inflammation (0-4)
1	0.75	0**	1**	0.75*	0**	1
2	0***	0**	1**	0**	0**	1
3	1	0**	1**	2	0**	1
4	2	1.75	2.25	3	2.25	1.75
5	1	2	2.5	2	3	2

(*): p<0.05, (**): p < 0.01, (***): p<0.001 when compared with groups 4 and 5 using Kruskal Wallis test and Dunn's multiple comparison test

scoring of stained liver tissues between immunized groups (1, 2 and 3) and groups 4 and 5 in most examined parameters (**Table 2**) .

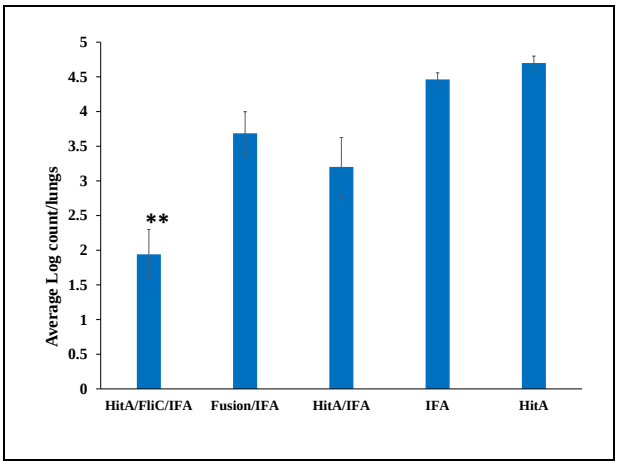

Figure 6. Lung bioburden following *P. aeruginosa* challenge. Lung bioburden was determined two days post-challenge. Error bars represent mean reading +/- SEM.

DISCUSSION

In this study, we examined the effect of recombinant flagellin and IFA on the vaccine protective efficacy of recombinant ferric iron-binding periplasmic protein, HitA. We recently showed that HitA is a promising vaccine candidate against *P. aeruginosa* infection when used in combination with BCG and IFA as immunoadjuvants[16]. Iron acquisition by *P. aeruginosa* is an important process for its virulence and survival in vivo[13]. Using antigens responsible for virulence as vaccine candidates is of dual benefit. First, once the immune system recognizes the virulence antigen, it neutralizes it resulting in loss of its virulence function. Second, the recognized antigen is used to facilitate the capture and opsonophagocytosis of the whole pathogen. In the current study, we initially confirmed the potential immunogenicity of HitA by antigenicity prediction algorithms and by western blot reactivity between HitA and mouse anti-*P. aeruginosa* serum antibodies.

IFA is well-known for its ability to enhance humoral antibody response with more bias towards T helper type 2 (Th2) immune response[25]. Flagellin is an effective immune stimulator of various biologic functions identified by Toll-like receptor 5 and nod-like receptor C4 (NLRC4)/neuronal apoptosis inhibitory protein 5 (NAIP5) inflammasome signaling[26]. It also promotes strong antigen specific T cell responses[27]. In addition, it stimulates cytokine production by a range of innate immune cells and triggers the recruitment of T lymphocytes to lymph nodes[9,26]. Due to these features, flagellin (FliC) of *Salmonella enterica* serovar Typhimurium has been reported as a promising immunoadjuvant. This was accomplished by either simple admixing or genetic fusion to vaccine antigens[28–30]. In the current study, we tried both approaches where we created a recombinant fusion protein (FliC-F-HitA) and tested its potential compared to simple admixing of FliC and HitA. Furthermore, we added IFA as an adjuvant due to its favorable depot effect that would be reflected on efficient antigen presentation and vaccine efficacy[31]. Our results demonstrated the beneficial effect of flagellin and IFA as immunoadjuvants where HitA-specific serum IgG antibodies were significantly elicited following immunization with HitA/FliC/IFA mixture, Fusion/IFA and also HitA/IFA. These antibodies enhanced opsonophagocytosis of *P. aeruginosa* by human PMN cells. Upon challenge of immunized mice, significant reduction in lung bioburden was mainly observed in the

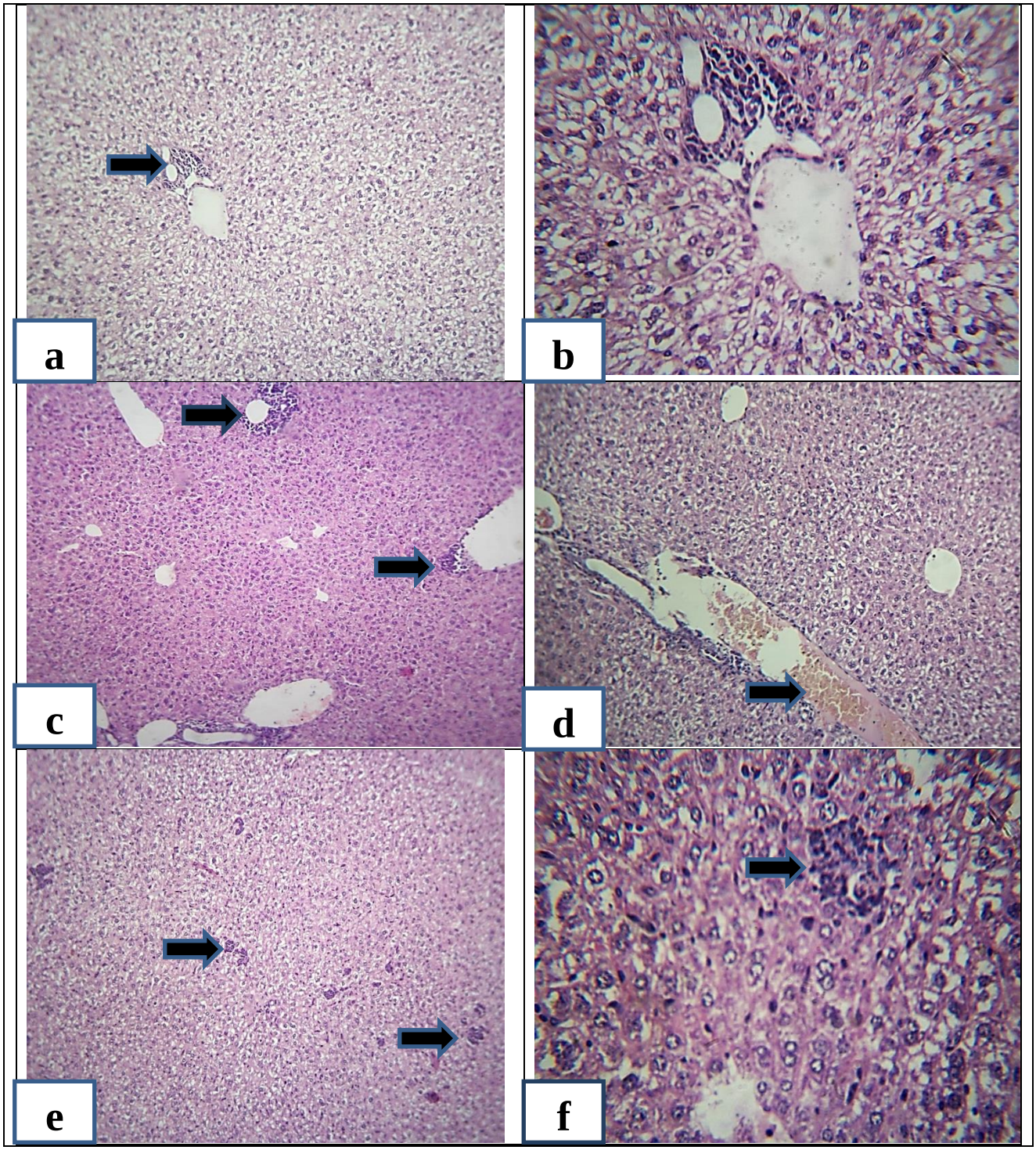

Figure 7. Histopathological examination of livers from different animal groups. (a) liver section in an immunized case (group 1) showing normal liver architectures, central veins with mild peri-portal inflammation (arrows) (x100). (b) a higher power view of the previous case showing mild focal peri-portal neutrophilic infiltration and mild diffuse micro-vesicular steatosis (x400). Liver sections in non-immunized case (group 4) showing (c): marked peri-portal inflammation, arrows (x100), (d): marked congestion, arrows (x100), (e): numerous foci of focal lytic necrosis; (f): higher power view of (e) showing the focal lytic necrosis where the necrotic hepatocytes are replaced by aggregates of neutrophils, arrows (x400).

group immunized with HitA/FliC/IFA mixture. The bioburden reduction was not statistically significant in lungs of other immunized groups (i.e. Fusion/IFA and HitA/IFA). A possible explanation of the failure of the Fusion protein (i.e. FliC-F-HitA) to reduce the lung bioburden might be the low immunizing dose given to mice. Fusion protein (FliC-F-HitA) was given at a dose of 10µg. Based on the molecular weights of FliC and HitA, the contribution of HitA in this fusion dose represented only 4 µg out of the 10µg injected. On the other hand, the other groups, HitA/FliC/IFA and HitA/IFA, were immunized with a higher dose of 10µg HitA. Using a higher dose of the fusion protein might stimulate a more protective efficacy. Furthermore, it was previously reported that the position of the antigen fused to flagellin played a critical role in immunogenicity outcome[9]. The antigen can be fused to the N-terminal or the C-terminal of flagellin or can be inserted into the hypervariable region of flagellin[9]. In our work, we fused HitA to the C-terminal end of flagellin. Trying the other two fusion options is worthy of further investigation. The failure of HitA/IFA, compared to HitA/FliC/IFA, to protect the lungs highlights the critical role of flagellin in augmenting the protective immune response. Investigation of detailed cytokine profile in the different animal groups should clarify the exact role/mechanism of flagellin immunostimulation. In our study, flagellin (FliC), whether fused or unfused, was administered at low doses (below 10 µg/mouse) as recommended by previous studies[9,28,29]. Low flagellin doses (1-10µg) were shown to be effective as vaccine adjuvant, reflecting its potent immunostimulatory effect[9,28,29]. Histopathological examination of livers of immunized mice revealed significant improvement when compared with livers of control groups. This was observed in most of the tested histopathological parameters (Table 2). It is noteworthy that group 5 immunized with HitA alone without adjuvants were not protected against challenge and no significant HitA-specific antibody response was observed. This was quite expected since it is well-reported that recombinant subunit antigens are weakly immunogenic and need immunoadjuvants to be effective as vaccines[8].

CONCLUSION

Overall, HitA ferric iron binding periplasmic protein is considered a promising vaccine candidate against *P. aeruginosa*. Flagellin, in combination with IFA, played an important role as immunoadjuvant to augment the immune response to HitA. Future studies combining HitA with other *P. aeruginosa* antigens could improve the vaccine protective outcome.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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