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Characterization of Immune Responses to *Yersinia pestis* (Indian Isolate) Infection in Mouse Model

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Abstract

Yersinia pestis, causative agent of plague, is one of the deadliest pathogens around globe. Innate immune response is first line of host defense against pathogens. Here, we have investigated innate and adaptive immune response in plague infected mice, gene expression levels of TLR1-9 and CD14, MyD88, NF-κB, TNF-α, MAPKp38, IL-1β were studied in peritoneal macrophages of plague infected mice in a time dependent manner (0 h, 24 h, 48 h, 72 h, 96 h and 120 h of post infection) by qRT-PCR. We also evaluated the immune response to Yersinia outer proteins (Yops) in *Y. pestis* infected mice. Selected genes (11 numbers) of *Y. pestis* encoding virulent factors viz, YpkA, YopH, YopM, V antigen, Pla, YopN, YopJ, YopE, YopK, F1 and pH6 antigen were amplified by PCR, cloned and expressed in *Escherichia coli*. To study the IgG and its isotypes level, ELISA and immunoblotting were performed using purified recombinant antigens. The major antigens recognized by murine plague infected sera were V antigen, YopH, YopM, YopE, F1 but very weak immunoreaction was observed in case of Pla. We observed a significant difference in IgG isotypes (IgG1, IgG2a, IgG2b and IgG3) to V antigen and F1, whereas in case of YopH and YopE (IgG1 and IgG2b) only. We also observed significant increase in the expression of CD14 at 48 h post infection, TLR4 and MyD88 at 72 h post infection in *Y. pestis* infected mouse peritoneal macrophages. Our findings suggest that both innate and humoral immune responses are crucial for protection.

Keywords: Plague; *Yersinia* outer proteins (Yops); Toll like receptors (TLRs); Innate immune response; IgG isotyping; *Yersinia pestis*

Introduction

Yersinia pestis, a Gram-negative bacterium causing plague is a biothreat agent [1,2]. It caused three massive pandemics in history and killed hundreds of millions of people [3]. If not treated with proper antibiotics, bacteria reaches lymph node, spread systemically through blood, and cause fatal sepsis [3]. Pneumonic form is difficult to treat because of speed of the disease's progress and by the time individuals are symptomatic, they are often close to death.

Yops are known as virulence determinants expressed by *Y. pestis* [4]. Yops are encoded on a 75-kb plasmid, *in vitro* expression from these genes and subsequent secretion and translocation by a Type III secretion system are regulated by some crucial factors like temperature, calcium, and host contact [5-9]. Majority of Yops play crucial role in determining the virulence of the bacteria. YopE indirectly depolymerizes actin microfilaments [8,10-12]; YopH, a tyrosine protein phosphatase [13,14]; YpkA, a serine/threonine protein kinase [15,16]; YopM is translocated through a type III secretion system (T3SS) into the host cell cytoplasm [17]. YopM of *Yersinia pestis* induces a global depletion of natural killer (NK) cells [18]. YopM does not have a known enzymatic activity and its mode of molecular action is still unknown [19]; and YopB, YopD, YopK, and YopN, involved in controlling Yop translocation [8,11,12,14,20-24]. V antigen has shown anti host function and played crucial role in the regulation of low calcium response [25,26]. There exists a second 100-kb plasmid which encodes a murine toxin and the capsular protein, F1 [27], necessary for full virulence [28]. Third plasmid of 10 kb encodes the bacteriocin, pesticin, and a plasminogen activator protease (Pla) necessary in most strains for virulence from a subcutaneous site [29]. Other factors involved in virulence include the pH6 antigen encoded by chromosome [30], and lipopolysaccharide (LPS). Earlier studies measured the immune response to *Y. pestis* infection with cell extracts of *Y. enterocolitica*, *Y. pseudotuberculosis*, or *Y. pestis* as the antigens [31-33]. Immune response to purified V antigen, YopM [34], and Pla [35] was reported for small numbers of cases of human plague. More recently immune response to purified antigens of *Y. pestis* including F1, V antigen, YpkA, YopH, YopM, YopB, YopD, YopN, YopE, YopK, plasminogen activator protease (Pla), and pH 6 antigen as well as purified lipopolysaccharide was reported in antibiotic treated experimental plague survived mice [36].

Pathogen-host interaction is an important aspect for understanding pathogenesis and consequently developing successful countermeasures. Normally, once the bacterium infects host, innate immune responses provide immediate protection and after 4-5 days, T-cell and B-cell mediated adaptive immune response begins to provide organism-specific protection. Adaptive immune response generates long-lasting immunological memory; as a result, the bacterium will be eradicated by the synergistic effect of both innate and adaptive immunity [3]. Here, we evaluated both innate and adaptive immune responses in experimental plague survived mice. Gene expression levels of TLRs (TLRs 1-9) and CD14, MyD88, NF-kB, TNF-α, MAPKp38, IL-1β were studied in the peritoneal macrophages of *Y. pestis* infected mice in a time dependent manner (0 h, 24 h, 48 h, 72 h, 96 h and 120 h) by qRT-PCR. We also evaluated immune response to 11 purified recombinant Yops and other

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antigens of *Y. pestis* including YpkA, YopH, YopM, V antigen, Pla, YopN, YopJ, YopE, YopK, F1 and pH6 antigen in experimental plague survived mice without any antibiotic treatment. This is the first report on Indian plague isolate (*Y. pestis*) to characterize in terms of protective immunity and immune response of infected mouse model.

Materials and Methods

Ethics statement

The animal studies experiments had an approval from the Institutional Animal Ethics Committee (IAEC) wide registration number 37/1999/CPCSEA and Institutional Biosafety committee (IBSC) wide reference no: MB/11/50/UT as per the institutional norms. The principles of good laboratory animal care were followed all through the experimental process. The mice were maintained in accordance with recommendations of committee for the purpose of control and supervision of experiments on animals, Govt. of India.

Bacterial strains and reagents

One of the virulent strains of *Y. pestis*, Antiqua biovar (S1 strain) recovered from an outbreak of primary pneumonic plague occurred in Northern India in 2002 [37,38] was subjected for this study. Brain Heart Infusion (BHI) media and agar were purchased from Himedia. Frozen stocks of *Y. pestis* were streaked on BHI agar plate and incubated at 28°C for 48 h. Single colony from BHI agar plate was further inoculated in 5 ml of BHI broth and grown at 28°C for 48 h, centrifuged, washed with BHI, CFU/ml was counted by plate count method. All live *Y. pestis* cultures and animal experiments were performed in BSL-3 facility.

Animal challenge with *Y. pestis* (S1 strain)

To determine LD_{50}, initially 10-fold serially diluted bacterial cultures were injected in Balb/C mice (n=30) by intra peritoneal route and the surviving rate of mice was observed up to 5 days of post infection. The LD_{50} was calculated by the method of Reed and Muench [39].

To study the innate immune response against *Y. pestis*, 45 BALB/c mice of 4-6 week old (Batch-I) were divided in infected group (n=30) and control group (n=15). Infected group was challenged with LD_{50} of *Y. pestis* (1×10^3 CFU/mouse) intraperitoneally and control group received only media. Three survived animals from each group of batch-I were sacrificed at 24 h, 48 h, 72 h, 96 h and 120 h of post infection. Peritoneal macrophages were isolated and pooled with respect to the time points, suspended in RNA later solution (Sigma, USA) and stored at -80°C for further use.

In another experiment (Batch-II) a group of 4-6 week old BALB/c mice (n=16) were used for challenge with LD_{50} of *Y. pestis* (1×10^3 CFU/mouse) intraperitoneally and a control group (n=4) received sterile PBS only. The survival of mice was observed from 24 h to 30 days. The 50% of the experimentally infected mice were found dead at 5 days of post infection. On day 0 and 22nd sera samples were collected from infected survived (n=8) and non-infected (n=4) mice.

Cloning, expression and purification *Y. pestis* antigens

The genes *ypkA, yopH, pla and lcrV, yopN, yopJ, yopE, yopK, caf1* and *pH6* were amplified by PCR and cloned into pET28a whereas yopM was cloned in pET30a (Novagen). The used primers details are given in Table 1. Cloning of each gene was confirmed by nucleotide sequencing (Applied Biosystem, USA). Recombinant proteins were expressed in BL21 (DE3) at 37°C after IPTG induction and purified in denaturing conditions (YpkA, YopH, and Pla, YopN, YopJ, YopE, YopK, F1 and pH6 antigen) whereas YopM and V antigen were purified in native condition by Ni-NTA columns (Qiagen) as described by manufacturer. The purified recombinant proteins were dialyzed and estimated by Bradford method [40].

SDS-PAGE and immunoblotting

Purified antigens (2 µg/lane) were separated by 10% SDS-PAGE. The gels were run containing purified recombinant proteins. The proteins on gels were visualized by staining with Coomassie blue to observe purity. The same set of antigens was transferred from gels on nitrocellulose membranes [41]. The membranes were probed at 1:1000 dilutions of pooled *Y. pestis* infected and non infected sera respectively. The membranes were incubated for 1 h at room temperature with

Gene	Oligos	Restriction sites	Amplicon size (bp)	Cloning vector	NCBI accession No
ypkA	For-5'-CATATGAAAAGCGTGAAAATCATGG-3' Rev-5'-GTCGACTCACATCCATTCCCGCTC-3'	Nde I Sal I	2199	pET28a+	AF074612.1
yopH	For-5'-ATACCATGGGCATGAACTTATCATTAAGCGATCTTCATC-3' Rev-5'-ATACTCGAGGCTATTTAATAATGGTCGCCCTTG-3'	Nco I Xho I	1407	pET28a+	AF053946.1
yopM	For-5'-GGTACCATGTTCATAAATCCAAGAAATGTATCTAA-3' Rev-5'-GTCGACCTACTCAAATACATCATCTTCAAGTTTGT-3'	Kpn I Sal I	1230	pET30a+	AF053946.1
lcrV	For-5'-CATATGATTAGAGCCTACGAACAAAAC-3' Rev-5'-GTCGACTCATTTACCAGACGTGTCATCTAG-3'	Nde I Sal I	981	pET28a+	NC 003131.1
pla	For-5'-ATACCATGGGCATGAAGAAAAGTTCTATTGTGGCAAC-3' Rev-5'-ATACTCGAGGAAGCGATATTGCAGACCCG-3'	Nco I Xho I	939	pET28a+	HM807367.1
yopN	For- 5'-CATATGACGACGCTTCATAACCTATC-3' Rev- 5'-GTCGACTCAGAAAGGTCGTACGCCAT-3'	Nde I Sal I	882	pET28a+	AF053946.1
yopJ	For-5'-CATATGATCGGACCAATATCACAAAT-3' Rev-5'-GTCGACTTATACTTTGAGAAGTGTTTTATATTCAGC-3'	Nde I Sal I	867	pET28a+	F074612.1
yopE	For-5'-CATATGAAAATATCATCATTTATTTCTACATC-3' Rev-5'-GTCGACTCACATCAATGACAGTAATTTCTG-3'	Nde I Sal I	660	pET28a+	NC_005813.1
yopK	For-5'-CATATGTTTATTAAAGATACTTATAACATGCGTG-3' Rev-5'-GTCGACTCATCCCATAATACATTCTTGATCG-3'	Nde I Sal I	549	pET28a+	AF053946.1
Caf1	For-5'-ATACCATGGGCATGAAAAAAATCAGTTCCGTTATCG-3' Rev-5'- ATACTCGAGTTGGTTAGATACGGTTACGGTTACAG-3'	Nco I Xho I	513	pET28a+	AF074611.1
pH6	For-5'-ATACCATGGGCATGAAAATGAAATGTTTTGCG-3' Rev-5'-ATACTCGAGAAATACATACTCTTCAACACGCC-3'	Nco I Xho I	477	pET28a+	NC_010159.1

Table 1: List of primers for cloning and expression of virulent genes of *Y. pestis*.

Oligos Name	Forward primer	Reverse primer	PCR Product (bp)	Accession number
TLR1	5'-ATGTGAGCTGAGGGACTTTG-3'	5'-GGATAGTGGAGACATGTGGAAG-3'	141	AF316985
TLR2	5'-ACTGTGTTCGTGCTTTCTGAG-3'	5'-ATGGCTTTCCTCTCAATGG-3'	139	AF124741
TLR3	5'-ATAAATGAGGACTTGCTGGAGG-3'	5'-TCAGGAAATTAACGGGACCAC-3'	114	AF355152
TLR4	5'-GAGGACTGGGTGAGAAATGAG-3'	5'-GTAGTGAAGGCAGAGGTGAAAG-3'	74	AF185285
TLR5	5'-AAGACTGCGATGAAGAGGAAG-3'	5'-CTGGATGGTCTTATAGCACAGG-3'	145	DQ414410
TLR6	5'-GTCAAGAACATAGGCTGGGTAG-3'	5'-GCAGAACAGTATCACAGGACAG-3'	138	AF314636
TLR7	5'-CACAGGCTCACCCATACTTC-3'	5'-ACTTCAGGTACCAAGGCATG-3'	141	AF334942
TLR8	5'-CTGGAGACACTGCTACTGAG-3'	5'-TCATCTTGGTTTGCAGGGAG-3'	141	AY035890
TLR9	5'-CTCCTTGATCTCCAACCGTATC-3'	5'-ACAGTTCCACTTGAGGTTCAG-3'	90	AF348140
CD14	5'-CTTTTCTCGGAGCCTATCTGG-3'	5'-CAACTTTCCTCGTCTAGCTCG-3'	138	BC057889
MyD88	5'-TCGAGTTTGTGCAGGAGATG-3'	5'-GCGACACCTTTTCTCAATTAGC-3'	136	U84409
NF-κB	5'-CACGGTTATGGGCAGGAAG-3'	5'-GCACCTTTGGATTCGCTTTG-3'	134	AY521462
TNF-α	5'-TTGCTCTGTGAAGGGAATGG-3'	5'-CCTGAGCCATAATCCCCTTTC-3'	140	NM013693
MAPKp38	5'-AGATGCTCGTTTTGGACTCAG-3'	5'-GGTCGTGGTACTGAGCAAAG-3'	80	AF195850
IL-1β	5'-ACGGACCCCAAAAGATGAAG-3'	5'-TTCTCCACAGCCACAATGAG-3'	138	BC011437
GAPDH	5'-CCCCAGCAAGGACACTGAGCAAG-3'	5'-TCTGGGATGGAAATTGTGAGGGAGA-3'	86	NT187045

Table 2: List of primers and the target sequences used for Quantitative real time PCR for analysis of TLRs and other cytokines in mice infected with *Y. pestis*.

polyclonal goat anti-mouse HRP conjugated IgG (Dako, Denmark) and the reaction was developed in DAB solution.

Indirect ELISA

To determine IgG titers in serum, an indirect IgG ELISA was performed. 100 ng/well of purified antigens including YopH, YopM, V antigen, Pla, YopE and F1 were individually coated into 96-well plates (Nunc, Denmark) in triplicates, reacted with sera samples (1:400 to 1:51200 in PBS). Subsequently plates were incubated with 1:1000 dilution of HRP-conjugated goat anti-mouse IgG. Plates were incubated for 1 h at 37°C and were developed with o-phenylenediamine (Sigma Aldrich, USA) and the absorbance was read at 492 nm by an ELISA reader (BioTeK, USA).

IgG Isotyping

In order to evaluate the circulating IgG isotypes, it was carried out for YopH, YopM, Pla, V antigen, YopE and F1 using isotype specific antibodies (goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 from BioRad, USA at 1:1000 dilutions) as described above in triplicates. Detection of bound isotype specific antibodies was done with peroxidase labeled rabbit anti-goat IgG (1:5000) and OPD substrate. The absorbance was read at 492 nm.

RNA isolation and cDNA synthesis

Total RNA was isolated from the peritoneal macrophages of *Y. pestis* challenged mice collected at different time point (0 h, 24 h, 48 h, 72 h, 96 h and 120 h) using GeneJET™ RNA Purification Kit (Fermentas, USA) as described by manufacturer. RNA was quantified in each sample by NanoDrop 2000C (Thermo Scientific, USA). RNA (100 ng) from each group was then reverse transcribed to cDNA using First Strand cDNA Synthesis Kit (Fermentas, USA) as described by manufacturer.

Quantitative real-time PCR

The qRT-PCR was performed in duplicates using primers designed by IDT SciTool using published target sequences. Primers were designed for TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, CD14, MyD88, NF-κB, TNF-α, IL-1β, MAPKp38 and the predicted product sizes are shown in Table 2. Real-time PCR was performed using CFX96 TouchTM Real-time PCR System (BioRad, USA) using the SsoFast Eva Green Supermix (BioRad, USA) SYBR green reagent as described by manufacturer. Briefly, 2 µl of cDNA was used for each PCR with 250 nM forward and reverse primers in a total volume of 25 µl. Thermal cycling conditions comprised 95°C for 30 sec followed by 45 cycles at 95°C for 5 sec. At the end, a melt curve analysis was done from 60°C-95°C. For normalized gene expression (ΔΔCq), the average threshold cycle (Cq) obtained in each PCR reaction was normalized against GAPDH reference gene as calibrator. The relative expression was analyzed and represented in terms of normalized fold expression with ± 1 standard deviation using CFX Manager™ software (BioRad, USA).

Statistical analysis

ELISA results for IgG and its subtypes have been presented as mean ± SE of triplicates of three individual experiments, and statistical comparisons were performed using SigmaStat4 Software (Aspire Software International, USA). The obtained mean OD values were analyzed to *t-test* and *P* values of <0.05 were considered as significant. The relative gene expression was analyzed and represented in terms of normalized fold expression by CFX Manager™ software (BioRad, USA).

Results

LD_{50} Determination

Using Reed and Muench method as described earlier calculated LD_{50} of Y. pestis in Balb/c mice. LD_{50} value was found to be 1×10^3 CFU/mouse as 50% of experimentally infected mice were found dead at 5 days of post infection.

Cloning, expression and purification of *Y. pestis* antigens

The genes *ypkA, yopH, pla, yopM, lcrV, yopN, yopJ, yopE, yopK, caf1* and *pH6* of 2199 bp, 1407 bp, 939 bp, 1230 bp, 981 bp, 882 bp, 867 bp, 660 bp, 549 bp, 513 bp and 477 bp encoding YpkA, YopH, Pla, YopM, V antigen, YopN, YopJ, YopE, YopK, F1 and pH6 antigen of 81.75 kDa, 50.88 kDa, 34.62 kDa, 46.21 kDa, 37.25 kDa, 32.68 kDa, 32.46 kDa, 23 kDa, 21 KDa, 17.67 kDa and 17.23 kDa molecular weight proteins respectively were cloned and expressed in BL21 (DE3) at 37°C. All recombinant proteins having histidine fusion tag were purified by Ni-NTA chromatography either in native or denatured condition. The yield of YpkA (20 mg), YopH (18 mg), Pla (10 mg), YopM (21 mg), V antigen (15 mg), YopN (21 mg), YopJ (17 mg), YopE (20 mg), YopK (18

mg) F1 (14 mg) and pH6 (13 mg) was obtained from one litre of shake flask culture of individual clone representing individual protein.

SDS-PAGE of purified *Y. pestis* antigens and immunoblotting

To determine molecular mass and purity, expressed recombinant proteins viz; YpkA, YopH, Pla, YopM and V antigen (Figure 1A) and YopN, YopJ, YopE, YopK, F1 and pH6 antigen (Figure 1D) were analyzed by SDS-PAGE. All recombinant proteins reported in present study represent similar molecular weight to those reported in literature. In a Western blot experiment, pooled sera of *Y. pestis* infected mice recognized the purified antigens include YopH, YopM, V antigen and Pla (Figure 1B); YopE and F1 (Figure 1E). Pre-infected pooled sera did not recognize any recombinant antigen (Figures 1C and 1F).

Humoral immune response to *Y. pestis* antigens in experimental plague surviving mice

IgG ELISA: On day 22nd of post-challenge, sera from infected mice were collected and analyzed by ELISA for IgG antibody response. Figure 3 shows immunological responses of pooled sera from 8 animals with *Y. pestis* antigens; immune response to one antigen tested (YpkA) was negative for pooled sera (data is not shown). Very good IgG antibody response to V antigen followed by YopM, YopH, and Pla was observed in plague infected mice sera when compared to pre-infected sera. Our results indicate that V antigen; YopE and F1 are the immunodominant antigens in magnitude of response, with titers ranging from 1:400 to 1:6400 (Figures 2C,2E and 2F). YopH and YopM were also observed immunodominant and titres were ranging from 1:400 to 1:3200 for both of the virulent factors (Figures 2A and 2B) respectively. Pla was found to be a weak immunogen with titres ranging from 1:400 to 1:3200 as observed high OD in control sera (Figure 2D).

IgG antibody isotyping: to observe pre-dominance of IgG isotypes in plague infected mice sera, the levels of IgG1, ELISA measured IgG2a, IgG2b and IgG3 for each recombinant antigen. In case of V antigen and F1, very high level of IgG1 followed by IgG2b, IgG3 and IgG2a was observed in plague infected sera with respect to pre-infected sera (Figures 3B and 3D). In case of YopH and YopE, IgG1 and IgG2b were significantly high in comparison to normal sera (Figures 3A and 3C). In case of YpkA, YopM and Pla, no significant difference was observed

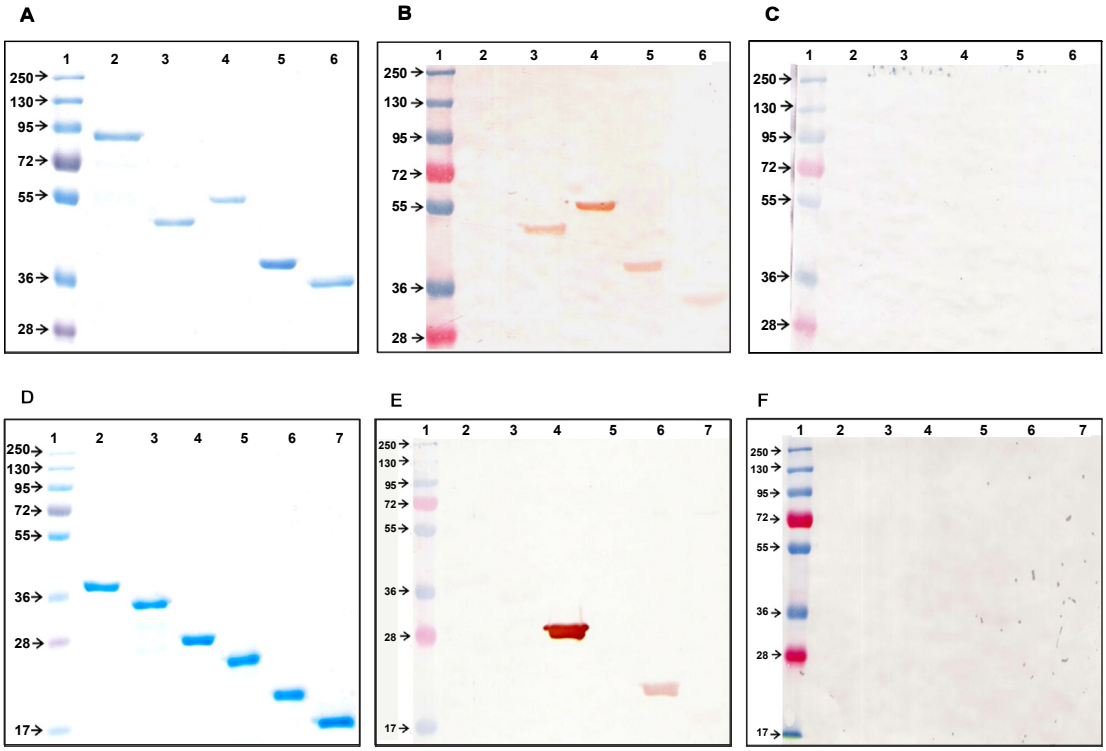

Figure 1: SDS–PAGE analysis of IMAC purified recombinant *Yersinia* Outer Proteins (YOPs) and immunoreactivity with *Y. pestis* infected mice sera. **[A]** The panel depicts Coomassie stained recombinant purified YpkA (lane 2); YopH (lane 3); YopM (lane-4); V antigen (lane 5) and Pla (lane 6). Protein molecular weight markers were run in lane 1. **[B]** Immunoblot analysis of IgG antibody response to Y. pestis recombinant antigens. The purified recombinant antigens (2 µg each) were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated with pooled sera (8 mice) at 1:1500 dilution with experimentally *Y. pestis* infected mice. The panel depicts YpkA (lane 2); YopH (lane 3); YopM (lane-4); V antigen (lane 5) and Pla (lane 6). Protein molecular weight markers were run in lane 1. **[C]** Immunoblot analysis of recombinant antigens with pre-infected mice sera at 1:1500 dilution. The panel depicts molecular weight markers (lane 1); YpkA (lane 2); YopH (lane 3); YopM (lane-4); V antigen (lane 5) and Pla (lane 6). **[D]** 12% SDS-PAGE analysis of recombinant YOPs. The panel depicts Coomassie stained recombinant purified YopN (lane 2); YopJ (lane 3); YopE (lane-4); YopK (lane 5) F1 (lane 6) and pH6 antigen (lane 7). Protein molecular weight markers were run in lane 1. **[E]** Immunoblot analysis of IgG antibody response to *Y. pestis* recombinant antigens. The purified recombinant antigens (2 µg each) were separated on 12% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated with pooled sera (8 mice) at 1:1500 dilution with experimentally *Y. pestis* infected mice. The panel depicts YopN (lane 2); YopJ (lane 3); YopE (lane-4); YopK (lane 5) F1 (lane 6) and pH6 antigen (lane 7). Protein molecular weight markers were run in lane 1. Their sizes (in kDa) are shown at the left of the panel. **[F]** Immunoblot analysis of recombinant antigens with pre-infected mice sera at 1:1500 dilution. The panel depicts protein molecular weight markers (lane 1); YopN (lane 2); YopJ (lane 3); YopE (lane-4); YopK (lane 5) F1 (lane 6) and pH6 antigen (lane 7).

(data not shown). The immune response of all 11 recombinant antigens has been summarized in Table 3.

Innate immune response of *Y. pestis* infected mice

To evaluate the innate immune response against *Y. pestis* infection, the expression levels of TLRs (TLR1 to TLR9) and other gene transcripts i.e., CD14, MyD88, NF-κB, TNF-α, MAPKp38, IL-1β were studied through qRT-PCR. As shown in Figure 4, all TLRs were detected with varied expression levels. Out of 9 TLRs tested, interestingly only TLR4 had a pronounced expression with 30 fold increase (Figure 4A). TLR2, TLR4 and TLR9 were also detected significantly in peritoneal macrophages. Relative expression of TLR1, TLR3, TLR5, TLR6, TLR7 and TLR8 was significantly lower as shown in Figures 4A and 4B. The elevated TLR4 at 72 h after infection showed nearly seven-fold drop after 120 h of post infection. No TLR4 expression was noticed after 120 h of post infection. Interestingly CD14 expression was seen prior infection (0 h) and its expression level raised more than 100 fold as early as 48 h of post infection. The CD14 level drastically reduced and no CD14 was detected at the end of 120 h of post infection as shown in Figure 4B. We also analyzed the expression of some adaptor molecules and cytokines, amongst which MyD88 showed a pronounced expression with more than 20 fold increase. IL1β and MAPKp38 genes were found to be down regulated as the infection progressed from 24 h to 120 h (Figure 4C). TNF-α level was found more than five-fold at all time points during the course of infection. However, a gradual decrease in expression level of TNF-α was noticed as infection progressed from 0 h to 120 h of post infection (Figure 4C). The NF-κB had no significant change in its expression level in response to *Y. pestis* infection.

Discussion

The innate immune response is a complex set of interactions that have evolved to optimize the response to pathogens. TLRs are transmembrane proteins expressed by cells of the innate immune system, which recognize invading microbes and activate signaling pathways that launch immune and inflammatory responses to destroy the invaders. In present study, we have investigated the innate immune response of experimental *Y. pestis* infected mice to gain insight into the TLRs response including CD14, MyD88, NF-κB, TNF-α, IL-1β, MAPKp38 gene transcripts which play the crucial role to develop the innate immune response during infection. This is the first report where nine TLRs (TLR1 to TLR9), CD14, MyD88, NF-κB, TNF-α, IL-1β and MAPKp38 have been studied in peritoneal macrophages isolated from experimental *Y. pestis* infected mice by qRT-PCR in a time dependent manner. Our results indicated that CD14 is the first receptor, which showed very high expression (100 fold) as early as 48 h of post infection. Interestingly, only TLR4 was observed showing pronounced expression

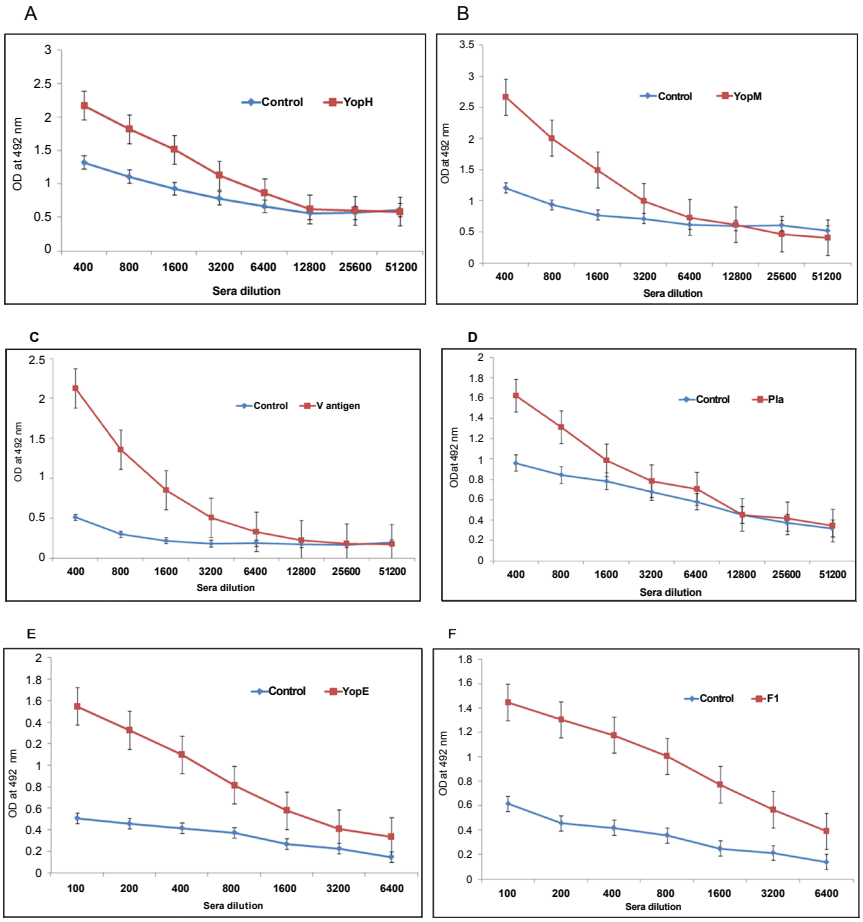

Figure 2: Indirect IgG ELISA titers for recognition of recombinant antigens; Infected sera were collected and pooled on 22nd day Y. pestis post challenge mice and for negative control Pre-infected sera were used. The Recombinant antigens [A] YopH; [B] YopM; [C] V antigen; [D] Pla; [E] YopE and [F] F1were tested for recognition at twofold dilution by ELISA.

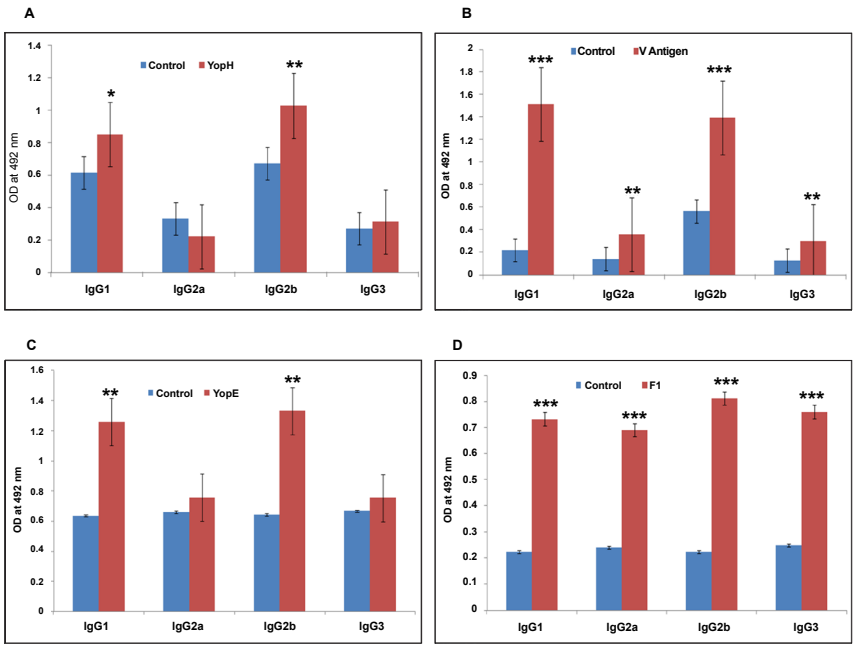

Figure 3: Profile of IgG subclass in sera from experimental plague infected mice. [A]. Sera samples collected on day 22 and were analyzed for presence of YopH-specific IgG1, IgG2a IgG2b and IgG3; [B] V antigen-specific IgG1, IgG2a IgG2b and IgG3; [C] YopE antigen-specific IgG1, IgG2a IgG2b and IgG3; [D] F1-specific IgG1, IgG2a IgG2b and IgG3 subclass antibodies by ELISA. The statistical comparisons were performed using Sigma Stat 4 Software (Aspire Software International, USA). *P<0.05; **P< 0.01. ***P<0.001. Analysis was done by *t-test*.

Name of recombinant antigen	Seroreactivity with *Y. pestis* infected mice sera	IgG titer in *Y. pestis* infected mice	IgG isotypes in *Y. pestis* infected mice
YpkA	No	NSD	NSD
YopH	Yes	1:3200	IgG1, IgG2b
YopM	Yes	1:3200	NSD
V antigen	Yes	1:6400	IgG1, IgG2a, IgG2b, IgG3
Pla	Yes	1:3200	NSD
Yop N	No	NSD	NSD
YopJ	No	NSD	NSD
YopE	Yes	1:6400	IgG1, IgG2b
YopK	No	NSD	NSD
F1	Yes	1:6400	IgG1, IgG2a, IgG2b, IgG3
pH6 antigen	No	NSD	NSD

NSD: No significant difference

Table 3: Summary of recombinant antigens of *Y. pestis* (Indian isolate) and their relative immune response in experimental infected Balb/C mice.

(30 fold) of 72 h post infection. We also observed that MyD88 an adaptor molecule showed more than 20 fold increased expression of 72 h post infection.

The signal transduction pathway for TLR4 activation by LPS (Lipopolysaccharide) serves as a representative example of the surface bound TLRs. LPS first binds to the CD14 receptor, which then transfers it to TLR4. TLR4 homodimerizes and forms a complex with the protein MD2. Cells need both MD2 and TLR4 in order to recognize LPS. TLR4 activation engages a set of MyD88 adaptor family members, including TIRAP, TRIF, TRAM (all three are TIR domains containing adapter proteins) and MyD88. This pattern of activation is general for cell surface TLRs, but the subsequent intracellular signal cascades, which include a number of transcription factor activations, are unique for each TLR. This results in a response that is appropriate to each threat [42]. Dziarski has proposed the TLR4 signalling pathway mechanism in case of *Y. pestis* infection that involves the activation of TLR4 by CD14 and subsequently activate the downstream molecule MyD88 and lead to the activation of cytokines and inflammatory pathways to eliminate the pathogen and provide immunity [43].Our findings are also in accordance with the above study.

Although there are few reports [30-32,37] on immune response to Yops, the novelty of our work lies in the use of 11 purified recombinant antigens (YpkA, YopH, YopM, V antigen, Pla, YopN, YopJ, YopE, YopK, F1 and pH6 antigen) for definitive conclusion without administration of antibiotics. Our results indicated the predominant IgG antibody response of mice surviving plague without any antibiotic treatment was against F1, V antigen, YopE, YopH, YopM, Pla. A significant increased antibody titer with predominance of IgG2b, IgG3 and IgG2a isotypes along with elevated level of IgG1 was observed to F1 and V antigen. In case of YopH and YopE, there was a significant increase in IgG1 and IgG2b. There are different subclasses of IgG immunoglobulins such as IgG1, IgG2a, IgG2b, and IgG3 that provide the immunity to most infectious agents. During a cell-mediated immune response, there is a gradual change in the predominant immunoglobulin class of the specific antibody produced. This isotype switch is controlled by T-cells and their cytokines. In mice, IL-4 generally switches activated B cells to the IgG1 isotype (Th2 type immune response); IFN-γ induces IgG2a and IgG3 responses (Th1 type) [43]. It is quite evident from these findings that *Y. pestis* infection in mice induces both Th1 and Th2 type of immune response that provide protective immunity in combination with innate immune response in plague survived mice.

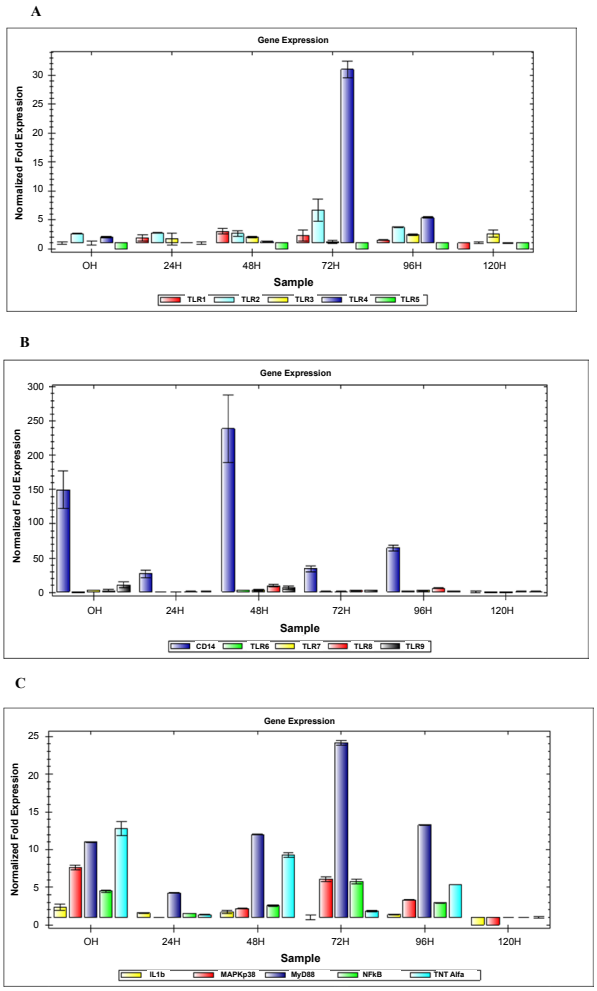

Figure 4: TLRs, CD14, MyD88, NF-κB, TNF-α, IL-1β, MAPKp38 expression in a time dependent manner in peritoneal macrophages of experimental plague infected mice. [A]. Fold change expression of TLR1 to TLR5. [B] Fold change expression in TLR6 to TLR9, CD14. [C] Fold change expression in MyD88, NF-κB, TNF-α, IL-1β, MAPKp38. The gene expression level was evaluated by qRT-PCR and normalized to the expression of the reference gene glyceraldehyde-3-phosphate-dehydrogenase. Fold change expression was analyzed and represented in terms of normalized fold expression with ± 1 standard deviation using CFX Manager™ software (BioRad, USA).

Earlier, the immune response was evaluated to Yops in plague survived mice treated with many antibiotics [36]. Here, our objective was to understand immune response to Yops in plague survived mice without any antibiotic treatment. Antibiotic treatment following infection may kill the pathogens and degraded products (exposed proteins/antigens) of bacteria including Yops evoke the host immune response. However, we have evaluated the immune response to Yops in experimental plague surviving mice, which may mimic natural scenario of plague infection survivals where no antibiotic treatment is given. Therefore, our findings suggest that the survival of mice following plague infection in present study was due to development of both innate as well as adaptive immunity. These findings may helpful to understand the host-pathogen interaction for development of new effective vaccine candidates.

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Conflict of Interests

All the authors have no conflict of interest to declare.

References

1. Bossi P, Bricaire F (2003) [The plague, possible bioterrorist act]. Presse Med 32: 804-807.
2. Broussard LA (2001) Biological agents: weapons of warfare and bioterrorism. Mol Diagn 6: 323-333.
3. Perry RD, Fetherston JD (1997) *Yersinia pestis* etiologic agent of plague. Clin Microbiol Rev 10: 35-66.
4. Portnoy DA, Wolf-Watz H, Bolin I, Beeder AB, Falkow S (1984) Characterization of common virulence plasmids in Yersinia species and their role in the expression of outer membrane proteins. Infect Immun 43: 108-114.
5. Cornelis GR, Wolf-Watz H (1997) The Yersinia Yop virulon: a bacterial system for subverting eukaryotic cells. Mol Microbiol 23: 861-867.
6. Forsberg A, Rosqvist R, Wolf-Watz H (1994) Regulation and polarized transfer of the Yersinia outer proteins (Yops) involved in antiphagocytosis. Trends Microbiol 2: 14-19.
7. Goguen JD, Yother J, Straley SC (1984) Genetic analysis of the low calcium response in Yersinia pestis mu d1(Ap lac) insertion mutants. J Bacteriol 160: 842-848.
8. Rosqvist R, Magnusson KE, Wolf-Watz H (1994) Target cell contact triggers expression and polarized transfer of Yersinia YopE cytotoxin into mammalian cells. EMBO J 13: 964-972.
9. Straley SC, Bowmer WS (1986) Virulence genes regulated at the transcriptional level by Ca2+ in Yersinia pestis include structural genes for outer membrane proteins. Infect Immun 51: 445-454.
10. Rosqvist R, Forsberg A, Rimpiläinen M, Bergman T, Wolf-Watz H (1990) The cytotoxic protein YopE of Yersinia obstructs the primary host defence. Mol Microbiol 4: 657-667.
11. Rosqvist R, Forsberg A, Wolf-Watz H (1991) Intracellular targeting of the Yersinia YopE cytotoxin in mammalian cells induces actin microfilament disruption. Infect Immun 59: 4562-4569.
12. Sory MP, Cornelis GR (1994) Translocation of a hybrid YopE-adenylate cyclase from Yersinia enterocolitica into HeLa cells. Mol Microbiol 14: 583-594.
13. Guan KL, Dixon JE (1990) Protein tyrosine phosphatase activity of an essential virulence determinant in Yersinia. Science 249: 553-556.
14. Persson C, Nordfelth R, Holstrom A, Håkansson S, Rosqvist R, et al. (1995) Cell surface bound *Yersinia* translocate the protein tyrosine phosphatase YopH by a polarized mechanism into the target cell. Mol Microbiol 18: 135-150.
15. Galyov EE, Håkansson S, Forsberg A, Wolf-Watz H (1993) A secreted protein kinase of Yersinia pseudotuberculosis is an indispensable virulence determinant. Nature 361: 730-732.
16. Håkansson S, Galyov EE, Rosqvist R, Wolf-Watz H (1996) The Yersinia YpkA Ser/Thr kinase is translocated and subsequently targeted to the inner surface of the HeLa cell plasma membrane. Mol Microbiol 20: 593-603.
17. Cornelis GR (2002) The Yersinia Ysc-Yop 'type III' weaponry. Nat Rev Mol Cell Biol 3: 742-752.
18. Kerschen EJ, Cohen DA, Kaplan AM, Straley SC (2004) The plague virulence protein YopM targets the innate immune response by causing a global depletion of NK cells. Infect Immun 72: 4589-4602.
19. Rüter C, Buss C, Scharnert J, Heusipp G, Schmidt MA (2010) A newly identified bacterial cell-penetrating peptide that reduces the transcription of pro-inflammatory cytokines. J Cell Sci 123: 2190-2198.
20. Forsberg A, Viitanen AM, Skurnik M, Wolf-Watz H (1991) The surface-located YopN protein is involved in calcium signal transduction in Yersinia pseudotuberculosis. Mol Microbiol 5: 977-986.
21. Håkansson S, Schesser K, Persson C, Galyov EE, Rosqvist R, et al. (1996) The

YopB protein of Yersinia pseudotuberculosis is essential for the translocation of Yop effector proteins across the target cell plasma membrane and displays a contact-dependent membrane disrupting activity. EMBO J 15: 5812-5823.

22. Holmström A, Rosqvist R, Wolf-Watz H, Forsberg A (1995) Virulence plasmid-encoded YopK is essential for Yersinia pseudotuberculosis to cause systemic infection in mice. Infect Immun 63: 2269-2276.
23. Holmström A, Petterson J, Rosqvist R, Håkansson S, Tafazoli F, et al. (1997) YopK of Yersinia pseudotuberculosis controls translocation of Yop effectors across the eukaryotic cell membrane. Mol Microbiol 24: 73-91.
24. Sory MP, Boland A, Lambermont I, Cornelis GR (1995) Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the cyaA gene fusion approach. Proc Natl Acad Sci U S A 92: 11998-12002.
25. Bergman T, Håkansson S, Forsberg A, Norlander L, Macellaro A, et al. (1991) Analysis of the V antigen lcrGVH-yopBD operon of Yersinia pseudotuberculosis: evidence for a regulatory role of LcrH and LcrV. J Bacteriol 173: 1607-1616.
26. Skrzypek E, Straley SC (1995) Differential effects of deletions in lcrV on secretion of V antigen, regulation of the low-Ca2+ response, and virulence of Yersinia pestis. J Bacteriol 177: 2530-2542.
27. Protsenko OA, Anisimov PI, Mosarov OT, Donnov NP, Popov YA (1983) Detection and characterization of *Yersinia pestis* plasmids determining pesticin I, fraction I antigen and mouse toxin synthesis. Genetika 19: 1081-1090.
28. Welkos SL, Davis KM, Pitt LM, Worsham PL, Freidlander AM (1995) Studies on the contribution of the F1 capsule-associated plasmid pFra to the virulence of Yersinia pestis. Contrib Microbiol Immunol 13: 299-305.
29. Sodeinde OA, Goguen JD (1988) Genetic analysis of the 9.5-kilobase virulence plasmid of Yersinia pestis. Infect Immun 56: 2743-2748.
30. Lindler LE, Tall BD (1993) Yersinia pestis pH 6 antigen forms fimbriae and is induced by intracellular association with macrophages. Mol Microbiol 8: 311-324.
31. Bölin I, Portnoy DA, Wolf-Watz H (1985) Expression of the temperature-inducible outer membrane proteins of yersiniae. Infect Immun 48: 234-240.
32. Drobkov VI, Marakulin IV, Pogorelsky IP, Darmov IV, Smirnov EV (1996) Antibody spectrum after the inoculation of *Yersinia pestis* and *Yersinia pseudotuberculosis* to susceptible animals. Zh Mikrobiol 2: 81-85.
33. Mazza, G, Karu AE, Kingsbury DT (1985) Immune response to plasmid- and chromosome-encoded *Yersinia* antigens. Infect Immun 48: 676-685.
34. Nemeth J, Straley SC (1997) Effect of Yersinia pestis YopM on experimental plague. Infect Immun 65: 924-930.
35. Easterbrook TJ, Reddin K, Robinson A, Modi N (1995) Studies on the immunogenicity of the Pla protein from Yersinia pestis. Contrib Microbiol Immunol 13: 214-215.
36. Benner GE, Andrews GP, Byrne WR, Strachan SD, Sample AK, et al. (1999) Immune response to Yersinia outer proteins and other Yersinia pestis antigens after experimental plague infection in mice. Infect Immun 67: 1922-1928.
37. Gupta ML, Sharma A (2007) Pneumonic plague, northern India, 2002. Emerg Infect Dis 13: 664-666.
38. Kingston JJ, Tuteja U, Kapil M, Murali HS, Batra HV (2009) Genotyping of Indian Yersinia pestis strains by MLVA and repetitive DNA sequence based PCRs. Antonie Van Leeuwenhoek 96: 303-312.
39. Reed LJ, Muench H (1938) A simple method for estimating fifty percent endpoints. Am J Hyg 27: 493-497.
40. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.
41. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76: 4350-4354.
42. Beutler B (2004) Inferences, questions and possibilities in Toll-like receptor signalling. Nature 8: 257-263.
43. Dziarski R (2006) Deadly plague versus mild-mannered TLR4. Nat Immunol 7: 1017-1019.
44. Roitt I, Brostoff J, Male D (2001) Immunology. (6th edn) Mosby Publications, London.