DISCUSSION
5. **Discussion**

Though the causative organism of anthrax, *Bacillus anthracis*, was first reported as early as later part of 18th century by Robert Koch, not much research has been done till the Second World War. During industrial revolution period, anthrax was nicknamed as Woolsorter’s disease since the disease was predominantly seen in the workers in wool industry. They were often exposed to the spores of the pathogen which would have arrived with the infected animals. Occasionally, the disease was reported in people who would have handled the anthrax infected animals or after consuming the anthrax infected animals. Leaving these, anthrax was never a disease as devastating as plague, cholera, or other communicable diseases in recent years owing to advances in antibiotic development, improvement in hygiene and advancement in veterinary sciences. However, it gained worldwide attention as a potential biological weapon agent during WW I and II. The impact of the biological weapons was not well noted then. But in later years, two anthropogenic incidents and some natural outbreaks have reset the focus on anthrax. Besides, anthrax still poses a serious problem around the globe especially in rural areas where hundreds of deaths get reported almost year. These incidents may not be comparable to the other deadly diseases such as malaria, cholera, tuberculosis or plague which take thousands of lives every year in developing and under-developed nations. Based on WHO estimations, 50 kg of anthrax spores released in a populace of 5 lakh, nearly 95,000 will be killed and additional 1.25 lakh infected within 2 hours. It takes nearly 60 days of rigorous antibiotic treatment and medical attention to the infected people to subside the infection. Besides, it can cause mass panic and disorder in the society. Adding to these, the initial symptoms of the inhalational anthrax resemble to typical flu-like and ingestional anthrax mimic gastro intestinal disorder which often mislead the diagnosis. Delayed diagnosis leads to severe toxemia and excess load of bacteria in the host system finally lead to death of the host in relatively short period of time. Added to this, is the lack of safe vaccines against anthrax disease for human use. Fortunately, vaccine is available for veterinary use which is derived from a live
noncapsulated Sterne 34F2 strain. Though effective, this attenuated spore vaccine shows reduction in potency and inconsistent variation in virulence which sometimes caused death in animals (Mock & Fouet, 2001; Wang & Roehrl, 2005). Hence fast, accurate and low cost diagnostic systems backed with safe vaccines are necessary to protect the cattle and humans from anthrax infection.

Early diagnosis of anthrax is critical as a sheer 24 h delay in the initiation of chemoprophylaxis can result in thousands of additional deaths and huge expenditure leading to an outbreak. Currently, the major components of the outbreak detection system around the world are 1) the clinical health care system, 2) State health departments, 3) academic organizations and 4) Collaborating non-governmental organizations. Traditionally, anthrax outbreaks were identified based on rapid reporting of ‘sentinel’ clinical cases from different aforementioned components. However, the severity of the disease and the potential likelihood of bioterrorism attack demand novel strategies for rapid detection of anthrax. Currently, Centers for Disease Control and Prevention (CDC) follows an advanced strategy of syndromic surveillance, where the pre-diagnostic data from the records of outpatient visits, pharmaceutical prescriptions and laboratory orders were constantly tracked for detecting disease outbreaks rapidly. This strategy, though very advantageous, is yet to be employed in the developing countries due to the huge cost involved (CDC invested >$75 million on BioSense syndromic surveillance system). Another drawback of syndromic surveillance is the ineffectiveness of the diagnostic approach as the symptoms of anthrax resemble much like of influenza such as headache, cough, malaise and if unchecked toxemia can occur leading to coma and death of the host. Hence, a non-syndromic diagnostic system including rapid reliable and sensitive pathogen detection platforms should be developed that can append the existing surveillance systems.

Added to this, specific detection of *B. anthracis* is complicated due to huge genetic admixture among the members of this group by horizontal transfer or occasional loss of the plasmids (pXO1 and pXO2) resulting in remarkable flux in the
phenotypes and genotypes (Zwick et al, 2012). Some *B. cereus* (Klee et al, 2010) and *B. thuringiensis* (Cachat et al, 2008) strains were found to possess pXO1 and pXO2 plasmids of *B. anthracis*. Owing to these facts, although many molecular methods viz., PCRs and immunoassays targeting pXO1 and pXO2 plasmid markers or spore capsule proteins were developed, the specificity of these targets is still questionable. Therefore, conventional identification by characteristic growth on specific media, colony morphology, non-motility, lack of hemolysis on blood agar plates and gamma phage lysis is still considered ‘gold standard’ in definite detection of *B. anthracis*. However, these tests are time consuming and labor intensive. It often takes 48 hours to arrive at conclusive results. Most of these methods work well with large quantity of the agent (Bell et al, 2002; Rao et al, 2010). During biological emergencies, the diagnosis has to be faster and reliable. Hence, the current research on development of diagnostic systems for anthrax around the world is targeted in improvement of the reliability and specificity of the existing sensitive PCRs, RT-PCRs and immuno assays (immuno capture, immuno fluorescence, immuno chromatographic and immuno magnetic) of various types. But again, the detection system solely dependent on plasmid borne genes may not be completely specific to the pathogen. Fully virulent *B. anthracis* strains possess two plasmids carrying virulent marker genes. Therefore, it is also imperative that a detection system should also report presence or absence of two plasmids in order to distinguish pathogenic and nonpathogenic strains of the pathogen.

Recently, a Surface-layer protein, Extractable Antigen 1 (EA1) is proven to generate antibodies that could specifically detect *B. anthracis* spores and vegetative cells, simultaneously despite the presence of its homologues in other *Bacillus* species (Love et al, 2008; Walper et al, 2012). This highly antigenic chromosomal borne EA1 is a more stable marker, as certain epitopes of this surface layer protein were found conserved and specific to *B. anthracis* (Love et al, 2008; Wang et al, 2009; Walper et al, 2012). The molecule has been shown to be highly antigenic protein present in vegetative cells and spore surface of *B. anthracis*. Though EA1 is a vegetative cell protein, it is found invariably on the spore wall as remnant
contaminant during sporulation. Unless washed thoroughly, EA1 cannot be shaken off from the spore wall. Therefore, we employed mAbs developed against EA1 for our immuno assays. Though the earlier reports have explored its possibility as a detection marker for B. anthracis, little work has been carried out on development of simple assays for cost effective detection systems. The EA1 based ELISA and IPCR developed in this study is sensitive and shows better specificity than routine ELISA based detection systems. Furthermore, the rapidity, ease of performance and the non-requirement of high-end instrumentation makes this assay advantageous in outbreak scenarios and for routine investigations.

The amino and carboxy terminal regions of EA1 were cloned and expressed separately to generate monoclonal antibodies and examined for their utility in specific detection of B. anthracis. The mAbs EAC108, EAC105 and EAC202 specifically reacted with B. anthracis in western blot and no cross-reactivity was observed even with B. cereus group that are closely related to B. anthracis and genetically almost identical to the pathogen. Further simple dot ELISA and sandwich ELISA methods were developed using mAb EAC108 for the easy detection of B. anthracis from blood and meat samples. The sandwich ELISA standardized in the present study allows direct detection of B. anthracis cells from sample enrichments from blood and food samples. Similarly, dot ELISA also allows detection of the pathogen from the culture isolated from food or clinical samples. EA1 has been reported to be present on spore surface as a contaminant and the mAbs generated against EA1 antigen were able to detect the vegetative cells and spores simultaneously. Immuno assays as stated above are cost effective, robust and simple to perform but are restricted to a certain level of sensitivity that may require additional steps of enrichment of bacteria which in turn delays the availability of results. Hence, we coupled ELISA with PCR to improve the sensitivity of the assay and differentiate pathogenic and non-pathogenic strains. We employed mAb to capture the spores or vegetative cells into microtiter wells. Then the DNA was extracted which was used for PCR. For PCR, we targeted pag and cap genes, encoding for toxin PA and capsule, representing pXO1 and pXO2 plasmids,
respectively. Extraction of DNA from spores pose another setback in these assays as the spores are very resilient to chemical detergents such as SDS, triton X-100 and others; resistant to heating and radiation. Various methodologies have been adopted for DNA release from within the spore, including autoclaving, sonication, bead beating, germination, chemical treatment and combination of these techniques. However, some of these techniques compromise the overall sensitivity of the detection system and these are more time-consuming (Levi et al, 2003; Aslan et al, 2008). Aslan et al (2008) have established microwave-accelerated metal-enhanced fluorescence (MAMEF) based extraction and detection of the pathogen in 60 seconds. In 2003, Levi et al reported bead beating technique employing FastDNA SPIN Kit for soil in which DNA was extracted in less than 3 min. However, sensitivity of PCR reactions was compromised since DNA extracted by bead beating resulted in sheared DNA. We followed a simple method of microwaving the spores for various time periods and compared the method with chemical treatment and heating. Simple microwave exposure for 60 sec at 900 W sufficed for extracting DNA from spores with greater efficiency. Increase in the exposure time to microwave irradiation increased the yielded of spore DNA.

Specificity and sensitivity are the two important criteria for any detection tool. These can be affected by many factors like high concentrations of competing antigens, DNA, salts, ionic detergents such SDS, and other organic compounds (Rao et al, 2010). In general, PCR is more sensitive than ELISA or any other immuno assay owing to the exponential amplification power of PCR. However, the major drawback associated with PCR is that it needs a clean starting sample and this technique is not suitable for detection of protein and non-nucleic acid containing samples. The sensitivity of conventional PCRs is greatly compromised in the presence of interfering components such as cell debris, media components or other substances. On the other hand, immuno assays can effectively detect the target molecules directly from the source and little they are affected by the presence of other substances in the test samples. However, immuno assays are less sensitive when compared to PCR. In general, for IPCR, target specific antibody is coupled with a
reporter DNA molecule like lambda DNA which is amplified using specific primers. In the current instance, certain modifications have been introduced. The antibody molecules were employed to capture the spores or bacterial cells onto the surface of the microtiter plates and the virulence marker genes of the pathogen were used as reporter molecules. Elimination of interfering substances can enhance the sensitivity of PCR. To achieve this, capturing of the target antigen step was introduced prior to PCR. When compared to conventional duplex PCR, the sensitivity of the IPCR was found to be 1000 times more when applied on mixed samples. Also, in the present study, we were able to differentiate pathogenic *B. anthracis* from vaccine strains which is very relevant especially in the context of false alarms during the biological emergencies. Besides, identification of extent of virulence of the pathogen can help in deciding the countermeasures to decontaminate the buildings exposed to anthrax attacks, thereby reducing unnecessary expenditure of effort and money on decontamination.

There are reports of multiplex PCRs for detection of *Bacillus anthracis*. Almost all of these studies have similar approaches. They have selected minimum three target genes: Two virulence markers from plasmids pXO1 and pXO2 and one marker from chromosomal DNA such as Ba813, *rpoB*, *gyrB* and 16S-23S ribosomal spacer regions (*Levi et al*, 2003; *Ellerbock et al*, 2002 and *Ko et al*, 2003). These approaches had some drawbacks especially nonvirulent strains lacking virulence plasmids or some *Bacillus sp.* phenotypically similar to *B. anthracis* may produce false-positive results (*Ko et al*, 2003). Besides, these assays are real time PCR formats which expect purity of the DNA sample. Though, in the present work, we deal with multiplex PCR targeting virulence genes and a chromosomal marker (EA1), the nonspecific organisms and other components interfering with PCR are effectively eliminated by simple immuno capture technique.

In a natural setup such as outbreak or *in vivo* infections in cattle or human, EA1 based techniques have potential applications. If these techniques were to be applied to detect the deliberately released spores in a bioterrorist attack, the
success of the results will be totally dependent on the purity of the spores. However, the results can still be achieved in such cases by germinating the spores in an enrichment media for one or two hours and then proceed to Dot ELISA, sandwich ELISA or IPCR. These developed immuno based detection systems can become effective tools in field application for anthrax investigations under any outbreak scenario.

Another important component for a prompt and effective response to anthrax related biological emergencies is a highly effective vaccine along with suitable treatment. Though the research for development of vaccine for anthrax dated back to 1930s, till date there are only two licensed vaccines available for human use, AVA and AVP which are cell free culture filtrate predominantly containing PA. The limitations of these vaccines have been elucidated in detail such as presence of sub optimal levels of EF and LF, require multiple boosters, fails to provide complete protection against inhalational challenge, and cannot provide protection against spores when present in high numbers (Aulinger et al, 2005; Wang and Roehrl, 2005). And the current vaccine research is mainly focused on PA and the other toxin components. These vaccines essentially target toxins and their role in controlling the multiplication of the pathogen is minimal. Hence, there is a need to develop more effective vaccines containing multi components such as spore antigens and S-layer proteins in addition to PA which are ideal choice for generating holistic protection targeting both toxins and bacilli. For instance, the immune response in the animals was improved by adding certain antigenic molecules to PA such as BclA (Brahmbhatt et al, 2007; Cote et al, 2012), ExsFA or p5303 (Cybulski et al, 2008; Cote et al, 2012) and EA1 (Uchida et al, 2012). But instead of mixing the different components, it would be easier to develop chimeric molecule which comprises all the components. Moreover, certain domains of these components would be sufficient enough to provide the optimum protection. For example, domain 4 of PA has the epitopes that were shown to protect the animals from toxin challenge (Flick-Smith et al, 2002; McConnell et al, 2006; Chichester et al, 2007; Park et al, 2008; Kaur et al, 2009). Therefore, in the present we attempted to
develop a bivalent chimeric protein, PE, which could assure dual protection against both the spore invasion and the toxin effects; and this was evaluated for protective efficacy both \textit{in vitro} and \textit{in vivo} models.

Balb/C mice were immunized with chimeric protein PE and a mixture of PA4 and EA1C (PAEA) for comparison with the well established immunogen, PA4. Both PE and PAEA displayed end point titers equivalent to that of PA4 at the end of immunization schedule. Earlier reports concluded that the presence of EA1 with PA did not adversely affect the ability of PA to induce protective immune response (Baillie et al, 2003) but, conferred better protection against lethal anthrax infection than immunization with PA alone (Uchida et al, 2012). Similarly we observed that the anti-PE and anti-PAEA antibodies provided nearly 80 % protection to RAW cell lines against LeTx, whereas, anti-PA4 conferred about 73% protection suggesting that the protection capabilities of PA4 were not affected but supplemented by addition of EA1 component. It also appears that in the chimeric protein, PE, the protective epitopes of PA4 and EA1C are functionally intact as in the native proteins. Isotyping studies of antisera raised against these proteins showed increased levels of IgG1 over IgG2a especially in mice that received the PA4 protein alone or as a component, as reported earlier (Wang et al, 2004; Kaur et al, 2009). Our observation reaffirms the association of IgG1 response with protective immunity against anthrax. Elevated IgG1 responses show strong polarity towards Th2 response which invokes humoral immune response and also activates CD+ T lymphocytes (Park et al, 2008). It could be further demonstrated that both the PE and PAEA induced humoral immune response mainly by activating CD+ T lymphocytes as shown in lymphocyte proliferation assay and this proliferation was higher in PE and PAEA when compared to PA4 or EA1C.

Though, PA had long been speculated as a passive contaminant found on the surfaces of spores further studies proved otherwise. Stepanov et al (1996) reported that sera from animals injected with noncapsulated toxin producing live vaccine strains exhibit activities directed towards inhibition of early stages of infection
specifically inhibition of spore germination and phagocytosis of spores. Similar results were shown later that the uptake of spores of fully virulent Ames strain and Sterne strain by macrophages was improved by incubating the spores with anti-PA antibodies followed by intracellular germination and subsequent killing which might contribute to a protective immune response (Welkos et al, 2001 and 2002) and a strong correlation has been established between the neutralizing titers and the survival rates in mice after toxin challenge (Gorantala et al, 2011). And it was found that the PA protein is made in the spores and translocated to the surface upon the exposure of spores to germinants (Welkos et al, 2005). And the phagocytic effects anti-PA antibodies appeared to be same for both ungerminated and germinated spores. In the present study as well, the anti-PE and anti-PAEA antibodies exhibited enhanced opsonization of spores by RAW cell lines better than that of anti-EA1C and anti-PA antibodies alone. Presentation of more epitopes by PE or PAEA to lymphocytes than individual molecules, PA4 and EA1C, could have contributed for this enhanced phagocytosis. We also observed that anti-PA4 antibodies has also displayed significant enhancement in opsonization of spores. So it is not surprising to observe anti-PA4 antibodies involving in opsonization of spores.

EA1, though, not a spore component, it persists passively on the spore wall firmly. Unless washed thoroughly, EA1 remains on the spore wall. It was proven through fluorescent microscopy that the antibodies against recombinant EA1 bind to spores (Uchida et al, 2012). They have also demonstrated that anti-EA1 antibodies could opsonize the spores. In the present study, we have also successfully demonstrated that anti-EA1 antibodies bind to vegetative cells and spore as well though the protein is bound passively to the spore wall. Therefore, anti-EA1 antibodies are capable of protection by enhancing the opsonization of spores and vegetative cells. Hence we observed certain levels of spore opsonization associated with anti-EA1C antibodies.
Domain 4 is the binding region of PA to host cells through which LF and EF gain entry into the host cells. Since the same domain of PA was integrated in PE or used along with EA1C in PAEA preparations, the antibodies against these proteins were hypothesized to neutralize the toxin effects. As expected, we found that the anti-PE and anti-PAEA antibodies were able to negate the effect of lethal toxin on RAW cell lines. We further assessed the toxin neutralizing capabilities of anti-PE and anti-PAEA antibodies in mice model. The crude toxin was able to produce edema in foot pads and necrosis in tissues such as liver, spleen, and intestine within 24 h when injected through intra peritoneal route. Except anti-EA1C, all other antisera efficiently controlled the effects of edema toxin. All the animals treated with anti-PA4, anti-PE, and anti-PAEA showed very little or no necrotic tissue damage when challenged with crude toxin. The antibodies raised against the domain 4 of PA which is present either in free form (PA4, PAEA) or fused with EA1C (PE) could inhibit the binding of PA toxin to the cell surface. Similar was the case when tissue damage was prominent in toxin challenged animals treated with anti-EA1C where anti-toxin antibodies were not generated.

In the present study, we could demonstrate that EA1 is a stable marker capable of generating specific monoclonal antibodies against the pathogen. The simple detection systems such as Dot ELISA, sandwich ELISA and IPCR developed based on EA1 are capable of detection of the pathogen in any form and any source with high sensitivity (10 CFU ml⁻¹ in IPCR). Concurrently, we could demonstrate that EA1 is a good immunogenic protein capable of eliciting immune response in mice models. EA1 in combination (fused or mixed) with PA4 was capable of providing high antibody titer, and proliferated the lymphocytes better than PA4 alone. We also showed that the anti-PE and anti-PAEA antibodies displayed better protection in controlling the edema induced by crude toxin. Passive immunization with anti-PE and anti-PAEA provided protection against toxin challenge in mice. The present study reveals that the chimeric protein consisting of heterologous regions of PA and EA1 can render better protection than PA4 or EA1C alone against toxins and bacilli. And further scope exists to explore this chimeric protein in higher animals.
for its protective potency and to develop test through different modes of vaccination along with various adjuvants or immunoboosters to further improve the vaccine efficiency and to reduce the dosage levels. Similarly, the specific mAbs against EA1 can be conjugated with fluorescent or nanoparticle probes to further improve the detection sensitivity.