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Comparative analysis of extractable proteins from *Clostridium perfringens* type A and type C strains showing varying degree of virulence

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ABSTRACT

The prevailing scenario of bioterrorism warrants development of medical countermeasures with expanded coverage of select agents. *Clostridium perfringens* is a pathogen of medical, veterinary and military importance, and has been listed as Validated Biological Agent. We employed 2DE–MS approach to identify a total of 134 unique proteins (529 protein spot features) from the extractable proteome of four type A and type C strains. Proteins showing altered expression under host-simulated conditions from virulent type A strain (ATCC13124) were also elucidated. Significant among the differentially expressed proteins were elongation factor, molecular chaperones, ribosomal proteins, carbamoyl phosphate synthase, clpB protein, choloylglycine hydrolase, phosphopyruvate hydratase, and trigger factor. Predictive elucidation, of putative virulence associated proteins and sequence conservation pattern of selected candidates, was carried out using homologous proteins from other bacterial select agents to screen for the commonality of putative antigenic determinants. Pathogens (17 select agents) were observed to form three discrete clusters; composition of I and II being consistent in most of the phylogenetic reconstructions. This work provides a basis for further validation of putative candidate proteins as prophylactic agents and for their ability to provide protection against clusters of pathogenic select bacterial agents; aimed at mitigating the shadows of biothreat.

1. Introduction

Clostridium perfringens is a pathogen of medical, veterinary, and military importance and has been listed as Validated Biological Agent [1]. This obligately anaerobic rod-shaped bacterium is a common inhabitant of gastrointestinal tracts of both animals and humans and widely distributed in soil and sewage. The five toxinotypes of *C. perfringens* are etiological agent for several diseases in humans and animals. The organism produces a myriad of toxins for disease manifestation and has been categorized into five toxinotypes (A through E) on the basis of their differential production [2]. The diseases caused by Type A strains include gas gangrene and gastrointestinal diseases in humans (food poisoning, antibiotic-associated diarrhea, sporadic diarrhea, sudden infant death syndrome), apart from diarrhea in animals [3]. *C. perfringens* type C strains cause necrotic enteritis in humans and animals, and enterotoxemia in sheep [2].

Gas gangrene, the most destructive of all clostridial diseases, is caused by Type A strains and is characterized by rapid destruction of tissue with production of gas [4]. The two World Wars witnessed death of hundreds of thousands of soldiers due to gas gangrene (accounting for approximately 1% of wounded personnel during World War II and 10% of wounded personnel during World War I) as a result of battlefield injuries and *C. perfringens* was widely recognized as the most important causal organism of the disease [5]. Mortality rates as high as 50%–80% for patients with gas gangrene from trauma or surgery has been reported in patients injured in natural hazards such as earthquakes [6]. The treatment of gas gangrene is extremely difficult as the infection becomes established in tissues that are deprived of blood. Vaccine development against gas gangrene has received little attention. During the two World Wars, therapeutic use of antisera, raised against toxoids of all five species of clostridia associated with gas

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gangrene, was shown to have some benefits if administered soon after trauma. Active immunization using inactive toxin moiety was demonstrated to yield positive results in animal model [7,8]. Importance of non-toxin protein antigens in disease expression, especially in colonization by the pathogenic bacteria, including *Clostridium difficile*, has been recently recognized [9,10]. Preparedness for bioterrorism or state sponsored warfare using biological agents requires development of effective pre-exposure prophylactic regimen as post-exposure therapies are plagued with antibiotic resistant strains. In the biothreat scenario the problems associated with vaccination is confounded by the number of agents and uncertainty of use and certainly, 'one vaccine-one pathogen' is not a pragmatic solution. The need for 'fostering the generation of new vaccines that could substantially broaden the spectrum of vaccine-preventable diseases' was recently highlighted by Cassonea and Rappuoli [11]. To identify common antigenic determinants is one way to broaden vaccine coverage at least to a group of infectious agents.

Bacterial surface proteins play an elementary role in the interaction between the bacterial cell and the host environment [12]. They are involved in adhesion to and invasion of host cells, in sensing the chemical and physical conditions of the external milieu and sending appropriate signals to the cytoplasmic compartment. Surface proteins play significant role for the interactions between bacterial and host cells and are hypothesized to contain components of effective vaccines [13,14]. However, increasing number of proteins, which are apparently not surface located, are shown to be immunogenic for several bacterial pathogens. The outer membrane proteins (OMPs) and the components of ABC transporter located in the periplasmic space or inner membrane are all demonstrated to be immunogenic in Gram-negative bacteria. There are many examples where convalescent antibody response has been observed in sera which are directed to cytoplasmic proteins [15–17]. This could possibly be due to surface localization of a proportion of the protein population on bacterial cell surface, as shown for some proteins such as Hsp60 [18,19]. It is also hypothesized that antigen-presenting cells (APCs) display cytoplasmic or periplasmic protein to the immune system from disrupted bacterial cell walls or those engulfed by APCs.

Elucidation of the proteins reported here, from strains differing significantly in imparting lethality in mouse model, is likely to reveal putative factors responsible for the host specificity and pathogenesis of the different *C. perfringens* pathovars. We also analyzed differential extractable proteome under host-simulated conditions by altering two regulators that trigger virulence-related protein expression in *C. perfringens* namely, deprivation of iron and addition of FBS in the growing media. This strategy has been used previously in comparative proteome analysis of some pathogenic microbes including *Bordetella pertussis* and *Yersinia pestis* [20,21].

The present investigation was carried out in the direction of developing strategies for detection of, and protection against, *C. perfringens* with the following objectives: (i) identification of dominant extractable proteins from *C. perfringens* type A and type C strains (2 strains from each type) showing differences in virulence in mouse model, (ii) elucidation of proteins from virulent type A strain (ATCC13124), showing altered expression under simulated host conditions, (iii) potential of these proteins as protein markers for detection and as putative vaccine candidates and predictive analysis with respect to their localization/function and (iv) sequence conservation pattern of selected candidates in homologous proteins, from other bacterial agents of military and human health importance, to screen for the commonality of motifs for selecting putative candidates likely to broaden the spectrum of coverage.

2. Materials and methods

2.1. Bacterial growth and culture conditions

C. perfringens ATCC13124 was procured from Becton Dickinson India Pvt. Ltd., India. Two type A strains of *C. perfringens* (AL08-13 and AL08-16) and two type C strains (AL05–1, H2-3, AG07-4, AG07-6, and AG07-9) were previously isolated from various environmental samples from northern India as described previously [10]. The cultures were maintained at 4 °C in cooked meat medium (CMM) containing 454 g of beef heart infusion, 20 g of proteose peptone, 2 g of dextrose, 5 g of sodium chloride, and 1000 ml of distilled water. For extraction of protein from the 4 strains of *C. perfringens*, cells were grown in modified reinforced clostridial medium (RCM) broth containing 10 g of peptone, 10 g of beef extract, 3 g of yeast extract, 5 g of dextrose, 5 g of NaCl, 3 g of cysteine HCl, 0.5 g of sodium acetate, and 1000 ml distilled water. All the media were procured either from Oxoid Ltd., England, or from Difco Laboratories, France, and were prepared anaerobically by standard methods using a gassing manifold and serum vials [22].

For differential expression of simulated *in vivo* and *in vitro* proteins analysis, *C. perfringens* ATCC13124 was first grown overnight in tryptose-yeast extract-glucose (TPYG) broth containing 50 g of pancreatic digest of casein, 5 g of peptone, 20 g of yeast extract, 4 g of glucose, 0.5 g of sodium thioglycollate, and 1000 ml of distilled water. Iron limitation and presence of serum (FBS) in TPYG medium is defined here as simulated *in vivo* condition as described by Eshghi et al. [23]. After overnight growth at 37 °C (corresponding to stationary phase of growth), cells were harvested by centrifugation at 10,000 × g for 10 min at 4 °C. Pellet was resuspended in 2 ml of PBS prepared anaerobically and inoculated in a) TPYG medium alone pre-incubated at 37 °C and b) TPYG medium depleted of iron by overnight incubation at 37 °C with 0.4 mM 2,2-bipyridyl and supplemented with fetal bovine serum (FBS) (Sigma, USA) to a final concentration of 5% (−Fe + FBS medium). The inoculated media were then incubated for an additional 6 h at 37 °C before harvesting cells for protein extraction.

2.2. Mouse lethality assay

Animal experiments were approved by the institutional Animal Ethical Committee at DRDE (protocol number BT-10/50/SIA), Gwalior and all efforts were made to minimize suffering of mice. *C. perfringens* cells were freshly grown in thioglycolate broth (TGB) containing 5 g of yeast extract, 15 g of tryptone, 5.5 g of glucose, 0.5 g of sodium thioglycolate, 2.5 g of sodium chloride, 0.5 g of L-cystine, 0.001 g of resazurin, 0.75 g of agar, and 1000 ml of distilled water.

Cells were harvested in late exponential phase growth at 37 °C and washed with anaerobically prepared phosphate-buffered saline (pH 7.2) before injection. In groups of 6 Female BALB/c mice (6–8 weeks old), 1.2×10^7 or 1.2×10^6 CFU were intraperitoneally injected. The number of cells (CFU) was determined by serial dilution plating on SPS agar as described previously [10]. Death was recorded intermittently and lethality was expressed as mean time to death.

2.3. Preparation of the extractable protein fraction

Extractable protein fraction was prepared by the method of Hansmeier et al. [24] with some modifications as described earlier [25]. Briefly, *C. perfringens* cells were grown on RCM or TPYG broth at 37 °C and 20 mL of culture was harvested in the exponential growth phase ($OD_{600\ nm}$–0.8). The harvested cells were washed twice with pre-cooled 50 mM Tris–HCl buffer, pH 7.2 and