CHAPTER 6

DISCUSSION

Despite the recent advances in neonatal research, sepsis is a life threatening disease condition in the newborns, in particular among preterms (Dombrovskiy, 2007). Sepsis accounts for 30-40% of total neonatal deaths each year in developing countries (Black *et al*., 2010; Das *et al*., 2011; Tripathi and Malik, 2010). Several adverse conditions like delivery at home, unhygienic practices, delayed diagnosis, overcrowding and understaffing at NICU, lack of proper knowledge about the importance of proper hygiene practices among staffs and workers of NICU aggravate the disease condition further in developing countries (Edmond and Zaidi, 2010; Zaidi *et al*., 2005; Pittet and Boyce, 2001).

A diverse spectrum of pathogens are implicated in sepsis of newborns, of which the contribution of Gram negatives are the most common, being responsible for 18-78% of all incidence of neonatal sepsis (Mutlu *et al*., 2011; Macharashvili *et al*., 2009; Kristof *et al*., 2009). *E. coli* is the most commonly associated Gram negative pathogen for neonatal sepsis in developing countries specifically in preterm and VLBW infants (Stoll *et al*., 2011; Weston *et al*., 2011).

Protease continues to be one of the essential VFs of pathogenic bacteria. Secreted microbial proteases may contribute in the pathogenesis by allowing penetration of the host tissue and subverting host's defence during the first phases of infection and hence demands immense attention. Secreted proteases also promote the pathogenesis of producer organism by exerting their effect on various intracellular targets. Although several VFs are being endorsed in the pathogenesis of NSEC isolates, however, there is scarcity of knowledge about the extracellular proteases among these isolates.

The success of a pathogen lies in its ability to survive, multiply and disseminate in the host. Several studies have shown that the SPATE proteins contribute to the success of

E. coli as a pathogen (Parham *et al*., 2005; Restieri *et al*., 2007; Spurbeck et al., 2012). SPATEs have been reported to be widely associated with different pathotypes of IPEC and ExPEC isolates such as those associated with UTI, pyelonephritis, cystitis. However, the association of SPATEs with NSEC isolates has not been explored earlier. This deserves attention as the contribution of SPATEs in the pathogenesis of NSEC would improve our understanding of a disease that causes considerable neonatal mortality and morbidity in developing countries. Though several VFs have been reported for NSEC isolates so far, no such can be considered as a definite marker for the diagnosis of the disease. Hence, the search for new VF for NSEC isolates may help us in identification of a specific diagnostic marker for the disease.

This study was initiated to examine the distribution of the SPATEs and their subtypes in NSEC isolates collected from three different centres. The prevalence of SPATE and different subtypes of SPATEs was found to be extremely high (89%) among the septicemic isolates. To rule out the presence of similar clones circulating within the unit and contributing to the high prevalence of SPATEs, PFGE was carried out. With the exception of few isolates, most of the isolates were diverse. Therefore, it can be concluded that the prevalence of SPATEs was not due to clonal relatedness of the isolates. To further corroborate the specific association of SPATEs with the septicemic isolates, we also determined the distribution of SPATEs among the fecal and environmental *E. coli* isolates. The significant differences in the occurrence of SPATEs between septicemic and fecal *E. coli* isolates (89% vs 7.5%) and between septicemic and environmental *E. coli* isolates (89% vs 3%) reconfirms the exclusive association of SPATEs with the NSEC isolates.

Vat was found to be the most prevalent (51%) SPATE among the NSEC isolates followed by Sat (39%). The presence of other SPATEs was also observed in the isolates but in fewer numbers. Vat and Sat are the two most extensively studied SPATEs among the ExPEC isolates. Several studies have evidenced the involvement of Vat in extraintestinal infections like UTI, cystitis, pyelonephritis and bacteremia (Parham *et al*., 2005; Messai *et al*., 2011). The role of Vat in promoting virulence was recognized when significant association of Vat was found with UTI isolates compared to commensal and diarrheagenic *E. coli* isolates (Restieri *et al*., 2007) and when UPEC isolates with Vat were found to be more likely to colonize the urinary tract (Spurbeck *et al*., 2012). Another autotransporter Tsh which is encoded on a plasmid that is prevalent in APEC isolates has both the protease and hemagglutinin activity. Studies have shown that Vat and Tsh share 78% amino acid identity for which Vat has previously been called Tsh (Restieri *et al*., 2007; Heimer *et al*., 2004). Interestingly, Vat (Tsh) was found to be expressed in mice during experimental infection in the urinary tract, as evidenced by RT-PCR (Heimer *et al*., 2004). Furthermore, it has been suggested that *vat* (*tsh*) delays neutrophil infiltration of the urinary tract in response to UPEC by cleaving surface glycoproteins from leukocytes that are involved in neutrophil attraction and migration (Ruiz-Perez *et al*., 2011). Vat was also found to be involved in the pathogenesis of APEC strains as a *vat* mutant was attenuated in a cellulite model of infection in chicken (Parreira and Gyles, 2003). Sat has also been found to be strongly associated with intestinal and extraintestinal infections. Concetta *et al*., reported the exclusive association of Sat with UTI isolates than with other clinical and commensal *E. coli* isolates (Restieri *et al*., 2007). Sat was found to cause vacuolation of bladder and kidney cells and was shown to promote tight junction lesions in DAEC isolates (Guyer *et al*., 2000).

An analysis of the different phylogroups of *E. coli* and the distribution of SPATEs in those groups was performed. According to previous studies, mostly phylogroup B2 and to a lesser extent group D are implicated in extraintestinal diseases including septicemia. In our study, we also found the predominant presence of these two phylogroups (B2 and D) among the septicemic isolates. Though 67% of the bloodstream isolates belonged to phylogroups B2 and D, what was interesting was the presence of A and B1 phylogroups in 33% of the septicemic isolates. Apart from a few exceptions (Abdallah *et al*., 2011; Usein *et al*., 2011), majority of studies claim that phylogroups A and B1 are more likely to comprise the commensal *E. coli* and lack most of the VFs (Duriez *et al*., 2001; Johnson and Russo, 2001). Further, a large proportion of the A and B1 isolates in our study (87%) possessed SPATEs. Though the proportion of these two phylogroups, A and B1 was also high among the fecal and environmental isolates, the presence of SPATEs, as already mentioned, was negligible among these two groups of isolates. This significant association of SPATEs with septicemic isolates made it imperative to look for the other VFs in the isolates.

Apart from *iucC*, all other virulence determinants were predominantly isolated from phylogroup B2. IucC is a siderophore required for growth in blood and iron-limiting environment. IucC is generally considered as a defensive VF which enhances the adaptability of the organism in adverse situations but is not directly linked to the causation of disease like the toxins or invasins (Kohler and Dobrindt, 2011). The bloodstream isolates of other phylogroups (A, B1, D) possessed few VFs though the prevalence of SPATEs was high in them. This suggested the association of SPATEs in the virulence of NSEC, the validity of which was subsequently reinforced by neonatal mice experiment. The animal assay served two purposes. It ruled out the contribution of *iucC* in active virulence as this gene was also evidenced in the fecal

isolates of phylogroups A and B1 and it consolidated the pathogenic potential of the bloodstream isolates belonging to phylogroups A and B1 possessing SPATEs. Thus it would appear that SPATEs contribute to the virulence of NSEC isolates. Though a closely related model has been used to assess lethality of ExPEC earlier (Picard *et al*., 1999), our study is unique in the use of 3-4 days mice for the lethality assay of septicemic isolates.

The expression of SPATEs was assessed by skim milk assay. Despite the detection of *spate* gene, 23 isolates did not show any proteolytic activity for the milk protein casein. This is not unusual as the expression or secretion of SPATEs depends on several factors. Moreover, due to the diversity of the passenger domain, SPATEs have different substrate specificities and casein is not a substrate for some of the subtypes of SPATEs like Pic, Tsh, Vat etc. Factors affecting the secretion of SPATEs from the outer membrane to the extracellular milieu could also be responsible for the absence of proteolytic activity on the plate, particularly, the role of the periplasmic chaperons that prevent the misfolding of the proteases cannot be ignored (Dautin, 2010). Another observation that was difficult to interpret was a positive result on the skim milk plate for six isolates harbouring only the *vat* gene. In an earlier study, it was hypothesized that due to the mutations in the first two amino acids in the GDSGSP conserved active-site serine protease motif of Vat, it was unable to cleave casein (Parreira and Gyles, 2003). If this hypothesis holds true then the observation in this study can be attributed to other unidentified SPATEs in those isolates.

In addition, the presence of multiple SPATEs in the isolates of phylogroup B2 was observed in this study, which actually re-establishes the virulence potential of this phylogroup. The analysis for the predominance of a particular combination of SPATEs among the septicemic isolates led us to identify the combination of Vat and

Sat as the most prevalent combination. This actually re-evaluates the dominance of these two subtypes of SPATEs among the septicemic isolates. This observation was supported by previous studies where both Vat and Sat was reported as the most predominant SPATEs for isolates causing UTI. For a few isolates no subtypes of SPATEs could be identified by PCR based screening, though the conserved C-terminal translocator β-domain was amplified. Limiting the detection to 10 subtypes of SPATEs in our study might be a probable explanation but the possibility of newer subtypes also needs to be explored.

One of the objectives of the present study was to look for the presence of other proteases among the NSEC isolates, if any. In search for a novel protease, we selected a strain that was positive for the *spate* gene as detected with primers for the conserved C-terminal translocator domain but negative for the tested subtypes of SPATEs. The culture supernatant of this isolate was positive for protease activity on the gelatin zymogram and using the pNA oligopeptide substrate assay. To our surprise, the SDS-PAGE zymogram revealed the presence of a high molecular weight protease, the molecular weight of which was much higher than any of the previously reported subtypes of SPATEs. Furthermore, inhibition of the proteolytic activity with various inhibitors showed that the protease was almost completely inhibited by EDTA and 1,10 Phenanthroline, indicating the presence of a Zn++-dependent metalloprotease. Proteases are typically classified into four groups based on the residue present at their active site. These include, metalloprotease, serine protease, cysteine and aspartic acid proteases. Metalloproteases are the most extensively studied group of protease and zinc metalloproteases possess a characteristic zinc binding motif HEXXHXXGXXH. The secreted metalloprotease was identified by MS/MS peptide sequencing and

showed homology with *E. coli* protease YghJ having putative M60 metalloprotease domain.

YghJ belongs to a large family of eukaryotic and prokaryotic proteins containing putative metalloprotease domains (Nakjang *et al*., 2012). It appears that the member proteins of this family bears a canonical HEXXH metalloprotease motif within a domain named M60-like pfam 13402 and this domain is shared by both the pathogens and commensal organisms that colonize mucosal surfaces (Nakjang *et al*., 2012). This bioinformatical analysis was further confirmed when YghJ was identified from an ETEC isolate as a secreted metalloprotease and has been shown to degrade major mucins (MUC2 and MUC3) in the small intestine to promote colonization of ETEC to intestinal epithelial cells, thereby facilitating the delivery of heat-labile toxin to specific receptors on epithelial surface (Luo *et al*., 2014). Futhermore, in another recent study on ETEC, YghJ was found to be distributed over a diverse phylogenetic lineage and was shown to be expressed during the course of infection of ETEC. This study establishes that YghJ is one of the proteins that triggers immune response to ETEC infection. YghJ along with EtpA (an extracellular adhesion), EatA (a mucin-degrading serine protease) and EaeH (an adhesin) are suggested as the protective vaccine candidates for ETEC strains (Roy *et al*., 2010).

In contrary to the study by Luo *et al,* we found the gelatinase activity of YghJ. The differences in the producer organism and assay condition might be attributed to this discrepancy of result. YghJ was found to be optimally active at pH 7 to 8 which supports the fact that YghJ can degrade small intestinal mucins. We elucidated the optimum temperature for activity of YghJ at 37°C to 40°C. This actually establishes the importance of this protease in terms of pathogenesis as human pathogens are

mostly mesophiles and the virulence determinants should work optimally at this temperature .

We next determined the distribution of YghJ among the isolates of septicemic and fecal *E. coli*. Our results showed that YghJ is widely distributed among both groups of isolates, although the prevalence is less among the fecal isolates compared to the septicemic isolates (78% vs 54%). This is similar with the previous results obtained by Moriela *et al*., where YghJ was found to be widely distributed among a diverse pathotypes of *E. coli* including ExPEC and commensal isolates. The occurrence of *yghJ* gene among the fecal *E. coli* isolates guided us to determine the expression and secretion of YghJ in both groups of isolates. Our data revealed that the expression and secretion of YghJ is significantly higher among the septicemic isolates compared to the fecal *E. coli* isolates (80% vs 33%). This is in contrary to the previous study of Moriela *et al*., which suggested that YghJ is expressed in the two commensal isolates tested but failed to be secreted from these isolates due to the truncated T2SS region adjacent to *yghJ*. Moreover, this study has confirmed that functional T2SS is indeed needed for the secretion of YghJ. In our study, the Western blot analysis of YghJ was carried out with culture supernatant of each isolate, confirming the secretion of YghJ also from commensal isolates but the prevalence was less as compared to the NSEC isolates. It is important to note that in their earlier study, Luo *et al*., has shown that *E. coli* commensal strains HS and Nissle 1917 secrete comparatively little YghJ compared to clinical ETEC isolates.

YghJ has also been identified as the most protective vaccine candidate against ExPEC that provided almost complete protection from sepsis and mortality in a mouse model of sepsis (Moriela *et al*., 2010). In our study, we identified, purified and characterized YghJ from the culture supernatant of *E. coli* isolate causing neonatal septicemia.

Sepsis is the systemic inflammatory response of host against an infection which involves the release of an array of endogenous mediators. Proinflammatory cytokines are one of the mediators attributed to play key role in pathogenesis of sepsis and are implicated in the mortality and morbidity associated with septic shock. The proinflammatory cytokines are considered to be one of the most important diagnosticmarker for early diagnosis of neonatal sepsis (Machado *et al*., 2014; Ng and Lam, 2010; Lam and Ng, 2008). The VFs of NSEC isolates responsible for stimulating the proinflammatory response in neonatal host has not been elucidated yet. Only the bacterial LPS is the known factor for activation the proinflammatory response of host so far. On the other hand, the mode of pathogenesis of YghJ in neonatal sepsis has not been explored yet. So, we accessed the role of YghJ in triggering pro-inflammatory response in neonatal sepsis.

Our study creates a platform to begin our understanding on the proinflammatory response of *E. coli* metalloprotease YghJ in neonatal sepsis. An analysis of the kinetics of a panel of cytokines allows us to characterize the proinflammatory response of YghJ in neonatal sepsis.

A common limitation of the process for expressing recombinant proteins containing His-Tag in *E. coli* hosts is the contamination of recombinant protein with endotoxin (Salek-Ardakani *et al*., 2002). Endotoxin or LPS is a major component of the outer membrane of Gram-negative bacteria for maintaining structural integrity of the bacteria and is composed of O-antigen, core polysaccharide and Lipid A. LPS is known to signal through multiple receptors including macrophage receptor CD14, Toll-like receptor-4 (TLR4) and myeloid differentiated protein (MD)-2 receptor complex, resulting in the production of proinflammatory cytokines and chemokines such as TNF-α, IL-1 and IL-6 (Ulevitch and Tobias 1995; Poltorak *et al*., 1998;

Medzhitov *et al*., 1997; Medzhitov and Janeway 1999; Fattori *et al*., 1994). Importantly, the Lipid A moiety which is a phosphorylated glucosamine disaccharide, is mainly responsible for the immunostimulatory effects (Galanos *et al*., 1985; Raetz and Whitfield 2002).

There are some methods to remove LPS from recombinant proteins of which a common one is the use of a natural cyclic cationic decapeptide, Polymyxin B (PMB). PMB, the *Bacillus polymyxa*-derived antibiotic binds with a very strong affinity to the Lipid A moiety of LPS, the component required for anchoring of LPS to bacterial membrane and hence neutralizes LPS and prevent its binding to TLR4 (Moore *et al*., 1986). Several studies have demonstrated that PMB could successfully block LPS-stimulated cytokine production (Cardoso *et al*., 2007; Van der Kleij *et al*., 2004). Studies have also evidenced that PMB can protect animals from the toxic effects of LPS and has been used to prevent septic shock (Ferrari *et al*., 2004; Cooperstock, 1974; Corrigan and Bell, 1971; Gerard *et al*., 1993). Hence, we have detected cytokine stimulation in presence or absence of PMB in this study to neutralize the effect of LPS in cytokine stimulation and to establish the role of rYghJ in the induction of different cytokines.

The use of PMB in cultures of murine macrophages stimulated with recombinant YghJ completely abrogated the production of IL-6 in our study. IL-6 is known to be induced by LPS, IL-1 and TNF-α and has various biological effects including activation of B and T cells and modulation of haematopoiesis (Borden and Chin, 1994; Scheller and Rose-John, 2006). However, in contrast to TNF-α and IL-1, the injection of IL-6 does not produce a sepsis like state (Preiser *et al*., 1991). Moreover, despite its proinflammatory properties, IL-6 has also been shown to promote anti-inflammatory responses. IL-6 inhibits the release of TNF-α and IL-1 (Schindler *et al*.,

1990), the two major cytokines produced significantly on stimulation with rYghJ in our study. Therefore, this could be a reason behind the downregulation of IL-6 by rYghJ.

Unlike TNF-α, IL-1α and IL-1β, the induction of IL-8 was downregulated in presence of PMB but not completely abrogated like IL-6. The level of secreted IL-8 was almost 11 fold higher on treatment with rYghJ in presence of PMB compared to the control of 0 hrs of treatment. IL-8 is a multifunctional cytokine with chemotactic actions, and belongs to a large group of cytokines named chemokines. IL-8 is a multifunctional cytokine and is involved in acute and chronic inflammatory processes (Heinzmann *et al*., 2004) and has potent proangiogenic properties in vitro and in vivo (Belperio *et al*., 2000). The induction kinetics of IL-8 differed from that of TNF-α, IL-1α and IL-1β. rYghJ induced IL-8 stimulation was optimum at 4 hrs of post-stimulation compared to the other cytokines which were optimally induced at 10 hrs of post-treatment. The existing difference in the kinetics of various cytokines induction actually reflects the functional diversity of these mediators. Our results showed that YghJ induced IL-8 production is dose dependent.

The induction of TNF-α, IL-1α and IL-1β have not altered to a greater extent in presence of PMB. TNF-α and IL-1 are the most extensively studied cytokines in sepsis pathophysiology and together with IL-1, TNF-α was one of the first mediators identified in inflammatory sites (Schulte *et al*., 2013; Schouten *et al*., 2008). TNF-α not only enhance the production of macrophages from progenitor cells (Fahlman *et al*., 1994), but also facilitates activation and differentiation of macrophages (Witsell and Schook, 1992), stimulates other immune and non-immune cells like neutrophils, endothelial cellsand stimulates the secretion of an array of downstream cytokines including IL-6, IL-8, macrophage migration inhibitory factor (MIF), reactive oxygen

and nitrogen species (Cohen, 2002; Machado *et al*., 2014). All these effects actually amplify the proinflammatory response in sepsis. Our results showed remarkably higher production of TNF-α compared to other cytokines tested. Earlier studies have evidenced that in neonates, high levels of TNF-α is related to the severity of the disease (Pickler *et al*., 2010). This actually brings into focus the potentially important role of YghJ to exacerbate the disease condition in neonates.

The kinetics of the induction of TNF-α was found to precede by 1-2 hrs compared to IL-1α, IL-1β and IL-8 which is in agreement with the previous clinical and experimental studies which reported that TNF-α is an early mediator of sepsis and hence the role of TNF-α as a reliable biomarker for early onset sepsis has been suggested (DeForge and Remick, 1991; Schouten *et al*., 2008; Schulte *et al*., 2013; Shannon *et al*., 2007). In contrary, the secretion of the members (IL-1α and IL-1β) of IL-1 superfamily was delayed by 1- 2 hrs relative to TNF-α. However, previous reports have indicated IL-1 as the proximal mediator of inflammation, being released in a timely manner similar to TNF-α (Dinarello, 1997; Schulte *et al*., 2013). The levels of both the cytokines IL-1α and IL-1β peaked at 10 hrs after treatment and subsequently both of them declined in due course of time. The detection of the members of IL-1 became difficult at later time periods may be due to the short half-life of these cytokines and their interaction with soluble receptors specific to each cytokine (O'Neill, 2008). IL-1 is released from macrophages and synergistically with TNF-α activates macrophages for the production of other downstream cytokines and induce a shock like state (Dinarello, 1997; Okusawa *et al*., 1988). Because of its unique ability to stimulate downstream cytokine cascade, TNF-α is considered to be a master regulator of inflammatory cytokine production (Parameswaran and Patial, 2010), while IL-1 is an important cytokine in proinflammation (Schulte *et al*., 2013).

IL-1 has been found to be significantly increased in most patients with sepsis and associated with severity of sepsis (Hack *et al*., 1989; Loisa *et al*., 2003).

Our results also showed that YghJ could not stimulate the production of IL-12 from mouse macrophages. According to previous studies, the role of IL-12 in sepsis remains controversial. It was reported that survivors from severe sepsis produce more IL-12 from LPS-stimulated peripheral blood mononuclear cells (PBMCs) than non-survivors (Stanilova *et al*., 2005). Similarly it has also been found that defect in IL-12 production in patients undergoing major visceral surgery increases the risk of sepsis (Weighardt *et al*., 2002). So, IL-12 has been suggested to be a defensive factor of the host in these studies. Keeping this in mind, we can conclude that the lack of YghJ-induced IL-12 production actually reflects the role of YghJ in promoting proinflammation in neonatal sepsis and inhibiting the production of such cytokines that may have a role in preventing inflammation.

Our study demonstrates that YghJ not only promotes the induction of proinflammatory cytokines but also prevents the production of anti-inflammatory cytokines. IL-10, which is produced by different immune cells including monocytes, macrophages, T lymphocytes and B lymphocytes is known to prevent the excess proinflammatory response during sepsis (Schultz *et al*., 2007). IL-10 not only suppresses the production of several proinflammatory cytokines, but also induces the production of IL-1 receptor antagonist protein (IRAP-1) and soluble TNFR, hence reducing circulating concentration of these cytokines. The reduced production of IL-10 in our study reinforced the role of YghJ in promoting proinflammation in neonatal sepsis.

In summary, our study is the first investigating the distribution of 10 different subtypes of SPATEs among clinical *E. coli* isolates causing neonatal septicaemia and defining SPATEs as an essential factor in conferring virulence of NSEC isolates. The extremely higher prevalence of SPATEs (89%) compared to the fecal (7.5%) and environmental *E. coli* isolates (3%) depicted the exclusive association of SPATEs with the NSEC isolates. Results obtained from PFGE further ruled out the possibility of similar clones contributing to the higher prevalence of SPATEs and established that the predominance of SPATEs among the NSEC isolates was independent of clonal relatedness of the strains. Furthermore, remarkably higher prevalence of SPATEs compared to the other virulence determinants studied even in the non-B2 phylogroups of NSEC isolates actually indicates the specific association of SPATEs with the pathogenic isolates of neonatal septicaemia and delineates SPATEs as the most discriminatory trait for these isolates. In addition, the lethality of the septicemic isolates possessing SPATEs but no other VFs except *iucC* in the suckling mouse assay undoubtedly indicates that under certain circumstances SPATEs may have relevant role in the pathogenesis of such isolates. The animal experiment also ruled out the involvement of *iucC* in active virulence as the fecal isolates harbouring neither SPATEs nor VFs except *iucC* appeared to be non-pathogenic in the neonatal mice. The presence of multiple SPATEs among the isolates of phylogroup B2 revalidates the pathogenic potential of this phylogroup. Interestingly, Vat was found to be the most predominant SPATE among NSEC isolates which corroborates with other previous studies that have evidenced the involvement of Vat in several extraintestinal diseases. Our current findings have created a ground of interest to investigate further the mechanism of pathogenesis of Vat in neonatal septicaemia. The inability to identify a particular subtype of SPATEs in a few septicemic isolates is also worthy of

comment as it gave us a hint of the possibility of some newer subtypes to be discovered. Our search for a novel protease from a NSEC isolate led us to explore the identification of YghJ, a metalloprotease of *E. coli* recently been demonstrated to be secreted from ETEC strains (Luo Q *et al*., 2014). Our results showed that though the presence of *yghJ* is considerable among the fecal isolates, it is not expressed and secreted substantially among these isolates (33%) compared to the septicemic isolates (80%). We report for the first time that recombinant YghJ exhibits cytotoxicity to murine macrophages and human intestinal epithelial cell lines and can stimulate the production of proinflammatory cytokines and also down-regulates the production of anti-inflammatory cytokines, indicating YghJ as one of the essential factors contributing to the pathogenesis of sepsis.