CHAPTER 2

REVIEW OF LITERATURE

2.1 Epidemiology

2.1.1 Global epidemiology of neonatal mortality and morbidity due to sepsis

World Health Organization (WHO) reported death of approximately 5 million neonates globally each year, 98% of which takes place in developing countries (WHO 1999). Sepsis is the major cause of neonatal mortality and morbidity across the globe, 30-50% of the total neonatal deaths occur due to sepsis in developing countries. According to WHO, out of these 5 million deaths, 1 million deaths per year (10% of all under –five mortality) are due to neonatal sepsis and 42% of these occur in the first week of life (Lawn *et al*., 2005). Sepsis accounted for more than 90% of neonatal deaths in the pre-antibiotic years but after the availability of antibiotics, it has been reduced to 10 to 50% (Gheibi *et al*., 2005; Yalaz *et al*., 2006).

A

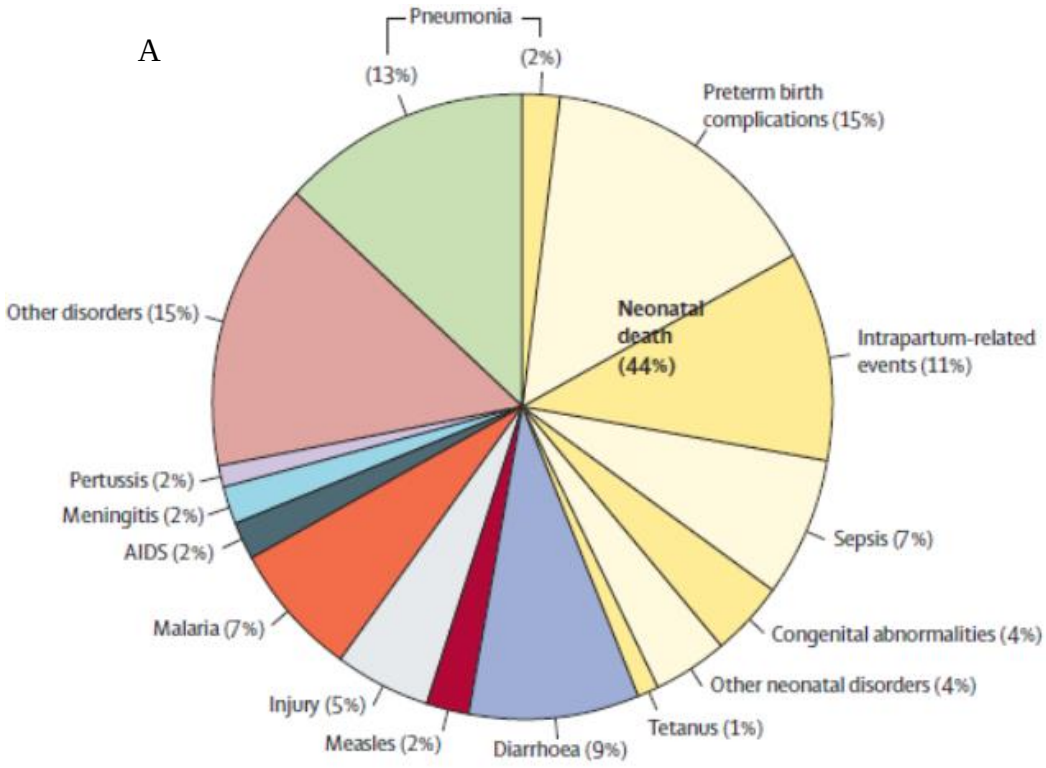

Fig. 2.1 A. Global causes of child deaths in 2013.

	Number of deaths (UR; millions)
Neonates aged 0–27 days	
Preterm birth complications	0·965 (0·615–1·537)
Intrapartum-related complications	0·662 (0·421–1·054)
Sepsis	0·421 (0·269–0·688)
Congenital abnormalities	0·276 (0·175–0·438)
Other disorders	0·232 (0·145–0·373)
Neonatal pneumonia*	0·136 (0·084–0·219)
Tetanus	0·049 (0·032–0·079)
Neonatal diarrhoea†	0·020 (0·012–0·033)

Fig. 2.1 B. Estimated number of neonatal deaths globally by cause in 2013. UR=uncertainty range (Liu *et al*., 2015).

In 2013, 6.3 million child deaths under 5 yrs were reported, of which 44% was found to occur in the neonatal period. The major causes accounted for the neonatal deaths in 2013 were preterm birth complications (0.965 million), intra-partum related complications (0.662 million) and neonatal sepsis (0.421 million) (Fig. 2.1) (Liu *et al*., 2015).

2.1.2 Epidemiology of neonatal mortality and morbidity in India due to sepsis

In spite of long standing commitment and advances in recent neonatal research, a high rate of child mortality exists in India. In India the estimated Under 5 mortaity rate (U5MR) is 69 per 1000 live births, placing the country at 49th position in terms of U5MR. A death of 1.84 million of children under five was reported in India, of which 78% occurred during first year of life, including 943,000 deaths in the neonatal period. Hence, neonatal deaths account for 65% of infant and 52% of under 5 child deaths. Of these, 81% of neonatal deaths occur within the first week of life. The major causes of neonatal deaths in India are: prematurity and low birth weight; neonatal infections including pneumonia, sepsis and CNS infections; and birth asphyxia and

birth trauma. Among these, sepsis and pneumonia stand as the commonest cause of neonatal deaths, being responsible for 30.4% of total neonatal deaths (Lahariya and Paul, 2010).

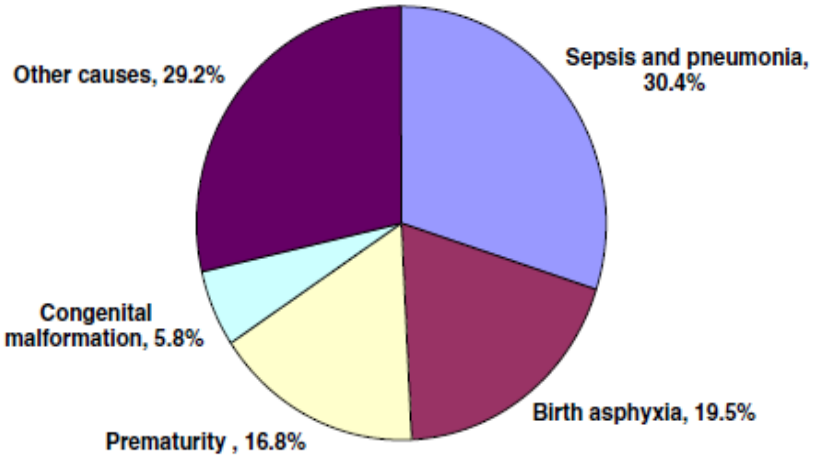

Fig. 2.2 Causes of neonatal deaths in India (Lahariya and Paul, 2010).

2.2 Classification of neonatal sepsis

Sepsis in newborns is broadly classified in two types based on the onset of sepsis, early onset sepsis (EOS) and late onset sepsis (LOS).

2.2.1 Early onset sepsis (EOS)

The EOS is more fatal than LOS, the neonate is at high risk of prolonged morbidity and mortality in EOS (Stoll *et al*., 1998; Chacko and Sohi, 2005). EOS presents within 72 hrs of life. In EOS, the organism is acquired from mother before or during delivery. In most of the cases, the pathogens residing in maternal genital tract infect the foetus through the prematurely ruptured or intact amniotic membranes (Kaftan and Kinney 1998).

2.2.2 Late onset sepsis (LOS)

LOS usually occurs after 72 hrs of life. The source of infection is normally nosocomial or community-acquired (Bizarro *et al*., 2005; Shah and Padbury, 2014). In

the last two decades, frequency of LOS has been reported to be higher compared to the EOS in NICUs (Fig. 2.3) (Sankar *et al*., 2008).

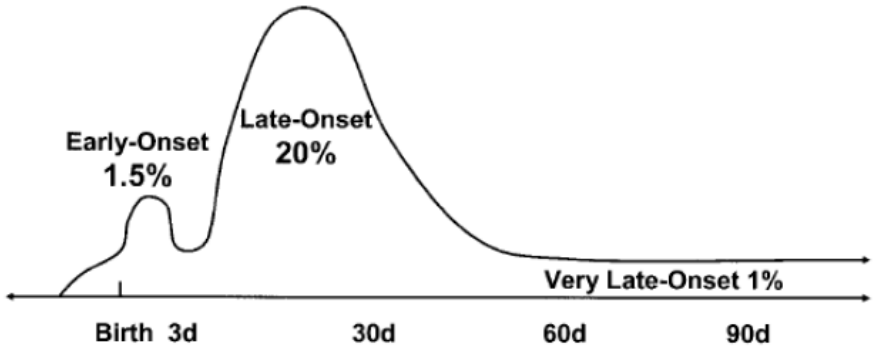

Fig. 2.3 Frequency of EOS and LOS in very low birth weight (VLBW) infants. The frequency of LOS is much higher than EOS in VLBW infants (Sankar *et al*., 2008).

2.3 Etiologic agents

A wide range of pathogens (both Gram-positive and Gram-negative) are implicated in neonatal sepsis. However, the spectrum of organisms for EOS and LOS differs between developed and developing countries. Organisms associated with early and late onset sepsis are listed in Table 2.1 (Camacho-Gonzalez *et al*., 2013). Organisms causing EOS usually have lower chances of being drug-resistant compared to LOS. However, in resource-poor settings, unhygienic labour rooms, and lack of aseptic techniques allow transmission of pathogens (Basu, 2014). In a study by Zaidi *et al*. it has been demonstrated that any infection in a hospital-born baby in a low-income country should be considered to be potentially hospital-acquired, even if the onset is within the first few days of life (Zaidi *et al*., 2005). This resembles the findings of other recent studies which have shown that multidrug-resistant organisms do cause EOS (Viswanathan *et al*., 2012). Gram-negative sepsis has recently been found to be predominant in several neonatal intensive care units (NICUs) (Kristóf *et al*., 2009;

Shah *et al*., 1999) specifically in developing countries (Bhat *et al*., 2011; Ganatra *et al*., 2010; Joshi *et al*., 2000). It has been found that Gram-negative organisms are responsible for 18%-78% of all neonatal sepsis and risk of death is higher with Gram-negative than with Gram-positive ones (Mutlu *et al*., 2011; Macharashvili *et al*., 2009). The reported incidence of Gram-negative sepsis in newborns was as high as 47.5% to 64% in a study in North India (Roy *et al*., 2002). In another study, sepsis by Gram- negative organisms (80.4%) was found to be significantly higher than sepsis by Gram-positive organisms (20.6%) (Kaistha *et al*., 2010).

Table 2.1 Organisms associated with EOS and LOS (Camacho-Gonzalez *et al*., 2013).

Early-Onset Sepsis (EOS)	**Late-Onset Sepsis (LOS)**
Group B *Streptococcus*	Coagulase-negative *Staphylococcus*
Escherichia coli	*Staphylococcusaureus*
Listeria monocytogenes	Enterococci
Other streptococci: *Streptococcus*	Multidrug-resistant g-negative rods (*E*.
pyogenes, viridians group streptococci,	*coli*, *Klebsiella*, *Pseudomonas*,
Streptococcus pneumoniae	*Enterobacter*, *Citrobacter*, *Serratia*)
Enterococci	*Candida*
Nontypable *Haemophilus influenzae*	

The organism most frequently associated in developed countries for EOS is Group B *Streptococcus* (GBS), with *E. coli* being the second most dominant organism (Baltimore *et al*., 2001; Bizzaro *et al*., 2005; Ganatra *et al*., 2010). Other organisms include coagulase-negative *Staphylococcus*, *Haemophilus influenzae* and *Listeria monocytogenes*. In developing countries, *E. coli*, *Klebsiella*, *Staphylococcus aureus*, *Acinetobacter*, *Pseudomonas* have been identified as the major causative agents for EOS (Karthikeyan and Premkumar, 2001; Zaidi *et al*., 2009). Coagulase negative

Staphylococcus (CoNS), predominantly *Staphylococcus epidermidis* is the most common cause of LOS. The other organisms implicated in LOS of neonates are Group B *Streptococcus*, *Enterococcus*, *E. coli*, *Klebsiella* and *Pseudomonas* (Bizzaro *et al*., 2005, Karlowicz *et al*., 2000). The majority of organisms that cause community acquired neonatal sepsis (CANS) are Gram-negative pathogens, specifically in developing countries. The most common ones in order are *S. aureus*, *E. coli* and *Klebsiella* species (Waters *et al*., 2011).

2.4 Risk Factors

The determination and understanding of risk factors is critically important to control an outbreak of infection in the NICU. Risk factors for EOS and LOS are listed in Table 2.2 (Camacho-Gonzalez *et al*., 2013).

Table 2.2 Risk factors for EOS and LOS (Camacho-Gonzalez *et al*., 2013)

Risk factors for the development of neonatal sepsis	
Source	**Risk Factor**
Early-onset neonatal sepsis	Maternal Group B streptococcal colonization Chrorioamnionitis Premature rupture of membranes Prolonged rupture of membranes (>18 h) Maternal urinary tract infection Multiple pregnancies Preterm delivery (<37 wk)
Late-onset neonatal sepsis	Breakage of the natural barriers (skin and mucosa) Prolonged indwelling catheter use Invasive procedures (eg, endotracheal intubation) Necrotizing enterocolitis Prolonged use of antibiotics H_2-receptor blocker or proton pump inhibitor use
Neonatal[a]	Prematurity • Decreased passage of maternal immunoglobulin and specific antibodies • Immature function of immune system

[a] Increases the risk for both early-onset and late-onset neonatal sepsis

2.4.1 Intrinsic risk factors

2.4.1.1 Neonatal factors

There are several neonatal factors that enhance the risk for sepsis. Immature adaptive immune system and poorly developed innate immune response being the most important factor (Levy, 2007; Lewis *et al*., 2001) followed by prematurity and low birth weight of neonates. The lower the birth weight of neonates, the higher is the incidence of sepsis. Several studies have evidenced a strong association of birth weight with the development of EOS and LOS (Gray, 2008; Cohen-Wolkowiez, 2009; Mutlu *et al*., 2011). Occurrence of sepsis has also been correlated with race and sex of newborns. Studies have shown that black preterm neonates have an increased incidence of sepsis, accounting for 5.14 cases per 1000 births with a case fatality rate of 24.4% (Weston EJ et al., 2011). The data from different studies have shown that the incidence of bacterial sepsis and meningitis is more predominant in males than in females, especially in case of Gram-negative enteric bacilli (Bassani DG et al., 2010).

2.4.1.2 Maternal factors

Maternal group B Streptococcus (GBS) colonization, premature rupture of membranes (PROM) of > 18 hrs, prolonged rupture of membranes, maternal urinary tract infection, chorioamnionitis are the preliminary factors that increase the risk for sepsis in neonates (WHO, 2003).

2.4.2 Extrinsic risk factors

All the treatments that a neonate receives are likely to present the higher risk of infection in the newborn. Neonates are subjected to scalp electrodes or percutaneous punctures for blood sampling. Scalp electrodes provide a portal of entry for maternal genital microorganisms. Preterm and sick neonates often require feeding by nasogastric tubes, which facilitates entry of microbes in the upper gastrointestinal

tract. Breast milk and formula feeds administered by continuous infusion remain at RT for several hrs, allowing microbes to proliferate in the reservoir or tubing during infusion (Mehall *et al*., 2002).

According to some studies, administration of drugs other than antibiotics also contributes to the increased risk of infection. Indomethacin is a drug that is used for the closure of a patent ductusarteriosusin neonates which has been found to be associated with an increased occurrence of sepsis and necrotizing enterocolitis when compared to newborns treated with surgery or other medications, the mechanism of which is poorly understood (Ojala *et al*., 2000; Major *et al*., 1994).

Intravenous administration of lipid to neonates has also been found to be potentially associated with bacteremia caused by CoNS. The role of lipid as a nutritional source for the bacteria, mechanical blockage of the catheter by deposition of lipid in the lumen, and effect of lipid emulsions on the function of neutrophils and macrophages may contribute to the increased risk of sepsis. In a study by Avila *et al*., it has been found that 85% of the CoNS sepsis in VLBW infants was due to the administration of lipid to neonates during hospitalization, hence administration of intravenous lipid was shown as the most important risk factor for the development of CoNS bacteremia (Avila-Figueroa *et al*., 1998; Freeman *et al*., 1990). Another recent study has found a strong correlation between ranitidine therapy in neonates admitted to one NICU and higher incidence of late-onset bacterial sepsis, the mechanism of which is unknown (Bianconi *et al*., 2007).

2.4.3 Environmental risk factors

The organizational and structural components of the NICU have been found to be potentially important risk factors for neonatal sepsis. Overcrowding, understaffing, improper ventilation are the preliminary factors contributing to the increased risk for

neonatal infection (Moore, 2004). In a study, an outbreak of *Enterobacter cloacae* infection was found to be associated with understaffing and overcrowding. The infection decreased from 5.8% to 1.8% after a move to a new NICU with more nurses and space per infant, more accessible sinks, and improved ventilation (Harbarth *et al*., 1999).

Unhygienic practices among staffs and workers of NICU stands as a potential reason for increasing the risk of infection in the newborns. Poor access to handwashing facilities due to overcrowding, irritant contact dermatitis associated with frequent exposure to soap and water, and lack of institutional commitment to good hygiene, each of these factors have contributed to the increased risk of infection in NICU (Pittet and Boyce, 2001).

In addition to these, developing countries have some other environmental risk factors which actually increase the propensity of infection in newborns. Majority of newborns in developing countries are born at home and at least half of neonatal deaths occur in home births. Unsafe birthing practices such as delivery onto an unsterile floor, unsterile cord cutting and extremely unsafe cultural customs such as spreading dung on the newborn's umbilicus are the key factors to enhance the infection rates in the neonates (Zaidi *et al*., 2005).

2.5 Clinical Manifestations

The earliest signs of sepsis are nonspecific and ill-defined which include difficulty in feeding, reduced movements, lethargy, vomiting. Other prominent features are fever, jaundice, respiratory distress, cardiac signs such as abnormal heart rate, apnea, diarrhea, abdominal distension, regurgitation, bleeding, skin issues like abscesses, petechiae, sclerema, purpuric lesions, umbilical redness and ecthyma (Griffin *et al*., 2007; Garcia and Nager, 2002; De Felice *et al*., 2002; Tripathi and Malik, 2010). The

body temperature of an infant with sepsis may be elevated, depressed or normal. It has been found that the term infants are more likely to have higher temperature than preterm infants (12% versus 1%), whereas frequency of hypothermia is higher in case of preterm infants (13% versus 3%) (Bonadio *et al*., 1990).

2.6 Diagnosis

2.6.1 Blood culture

Blood culture is the gold standard to diagnose neonatal sepsis. The blood should be collected asceptically as early as possible in sepsis and positively before initiation of antibiotic therapy from fresh arterial or venous puncture (Paolucci *et al*., 2012). In an earlier report by Kellogg *et al*., a sample volume of 6 ml was recommended to optimize the sensitivity of the test (Kellogg *et al*., 1997). However, this amount represents approximately 4.5% of the total blood volume of neonates and hence many other studies suggested to take 1 ml of blood in a bottle containing 5-10 ml of culture media and recommended to use the whole volume for aerobic cultures, since anaerobic organisms are rare in NICU (Benitz, 2010; Schelonka *et al*., 1996). Blood cultures should be observed for at least 72 hrs before final interpretation, though it is now possible to detect bacterial growth at a concentration of 1-2 colony-forming unit (cfu) per ml within 12-24 hrs by recent advanced techniques such as BACTEC and BACT/ALERT blood culture systems (Tripathi and Malik, 2010). Limitations of blood culture include less sensitivity, false positivity of culture due to contamination with CoNS, reporting delay of 24-72 hrs (Paolucci *et al*., 2012). Some organisms such as *Neisseria meningitidis* and *Candida albicans* are likely to be present even in case of a healthy-looking neonate, this makes the interpretation of positive results challenging (Buttery, 2002). Although the recent improved blood culture systems are capable of saving time with their continuous blood culture monitoring systems,

subcultures are required for specific biochemical or other assays for proper identification of pathogens (Paolucci *et al*., 2012).

2.6.2 Urine culture

The incidence of positive urine culture in neonates with early onset sepsis is quite less. Due to lower sensitivity and higher costs of specimen processing, urine culture is not generally considered as a useful diagnostic tool for the detection of early onset sepsis in newborns. However, a suprapubic bladder puncture sample or bladder catheterization sample has been suggested in all cases of LOS (Tripathi and Malik, 2010).

2.6.3 Haematological markers

Various haematological markers such as white blood cell count and absolute neutrophil count have been proposed and validated as one of the diagnostic tools for neonatal sepsis, specifically if merged with the interpretations of other tests (Gerdes, 2004). This test alone is not recommended as a method for diagnosis of neonatal sepsis in large clinical surveys due to several reasons as different other conditions such as post gestational age, asphyxia, maternal factors like fever and hypertension can influence the expression of hematological markers (Chiesa *et al*., 2004).

2.6.4 Biomarkers

Newer advanced methods for diagnosing neonatal sepsis includes the assessment of biomarkers, though these have not yet been validated adequately in clinical trials of neonatal sepsis. Several limitations of culture-proven sepsis actually lead to screen for different biomarkers as a more potential, reliable and time saving method (Reinhart *et al*., 2012).

2.6.4.1 C-reactive protein (CRP)

CRP is a very useful early diagnostic marker for neonatal sepsis. CRP is produced by

liver within 6 to 8hrs of exposure to an infection or tissue damage, with both proinflammatory and anti-inflammatory effects (Hofer *et al*., 2012; Reinhart *et al*., 2012). It has a half life of 19 hrs and can increase more than 1000 fold during an infective process. Hence, it is very useful as an early diagnostic marker with a value of 5 mg/L. The specificity and positive predictive values for CRP are 96% and 87% respectively but the sensitivity is quite low (41%) (Tripathi and Malik, 2010). CRP identifies and binds with the infective agents and damaged tissues to eliminate them through interactions with inflammatory cells and mediators. However, CRP also prevents the adhesion of neutrophils to endothelial cells, inhibits superoxide production, and increases IL-1 receptor antagonist production (Gabay and Kushner, 1999).

2.6.4.2 Procalcitonin (PCT)

PCT is a prohormone of calcitonin but the induction of this prohormone during sepsis and other infections is regulated in a manner different from the hormonal activities of mature hormone (Sexton *et al*., 2008). The induction of PCT begins after an exposure to bacterial endotoxins or to mediators (IL-1β, TNF-α, IL-6) released in response to bacterial infections (Gogos *et al*., 2000), with a maximum rise at 6 to 8 hrs and remains for at least 24 hrs. Thus, the kinetic profile shown by PCT is more advantageous than CRP and cytokines. It is released by hepatocytes and monocytes. PCT has a half life of 25-30 hrs (Tripathi and Malik, 2010). Several studies have claimed that PCT with a value of> 2.3 ng/ml is a valuable biomarker for diagnosis of neonatal sepsis after CRP with a specificity and positive predictive value of 97% and 91% respectively. However, PCT levels can also be elevated in several other conditions such as stress, after surgery or in patients with cardiac shock or autoimmune diseases (Cetinkaya *et al*., 2009; Turner *et al*., 2006).

2.6.4.3 Serum Amyloid A (SAA)

In pre-term neonates SAA was found to be a very essential and sensitive marker for rapid diagnosis and follow-up of neonatal sepsis. It is synthesized at the very onset of inflammation and hence a reliable marker for early diagnosis of sepsis. It is therefore in use in combination with other sepsis markers such as CRP and PCT for diagnosis of sepsis in newborns (Cetinkaya *et al*., 2009).

2.6.4.4 Liposaccharide-binding protein (LBP)

LBP binds to LPS of G-negative bacteria to form LPS-LBP complex (Wurfel *et al*., 1994) which actually binds to CD14 and TLRs to facilitate signal transduction leading to the activation of mitogen-activated protein kinase and nuclear factor κβ. Normally LBP is present in human serum at a basal level of 5-10 µg/ml, but this increases up to 200 µg/ml during the acute phase reaction (Tobias *et al*., 1992).

2.6.4.5 Cytokines

Cytokines are endogenous immunomodulating chemical agents and are secreted as a part of innate immune response of the host. They are regulated by a complex web of regulatory mechanisms including different cell types (Hotchkiss and Karl, 2003). In sepsis, both pro- and anti-inflammatory cytokines are secreted simultaneously by endothelial/epithelial cells and tissue macrophages immediately after an infection (Tang *et al*., 2010). IL-6 and IL-8 are the two mostly elevated pro-inflammatory cytokines in neonatal sepsis. Umbilical cord blood IL-6 has been shown to be a sensitive marker for diagnosing early onset sepsis, but sensitivity decreases at 24 and 48 hrs as IL-6 concentration fall rapidly and become undetectable after 24 hrs (Mehr and Doyle, 2000). IL-8 has similar kinetics with IL-6, with sensitivities from 80% to 91% and specificities from 76% to 100%. In a recent trial of 1291 clinically stable infants with clinical signs or obstetric risk factors suggesting early onset neonatal

sepsis revealed that the combination of IL8>70 pg/ml and/or CRP>10 mg/l significantly reduced antibiotic therapy from 49.6% to 36.1%, sensitivity was 80%, specificity 87%, positive predictive value 68% and negative predictive value 93% (Franz *et al*., 2004). The limitations of IL-6 and IL-8 must be taken into account. Both these cytokines can also be induced in case of trauma and major surgery, during acute exacerbations of autoimmune disorders, viral infections and after transplant rejection. Despite their role in pathogenesis of sepsis, the role of cytokines as sepsis biomarkers remains to be established as they are also produced in several non-infectious diseases as well (Robak *et al*., 1998; Malaguarnera *et al*., 1997; Nast-Kolb *et al*., 1997).

TNF-α is another proinflammatory cytokine that stimulates IL-6 production and acts broadly on several types of immune and non-immune cells. TNF-α level has been found to be higher in neonates with early onset sepsis than in non-infected ones. In a recent study, it has been shown that both TNF-α and IL-6 are higher in patients with sepsis than in controls. The optimal cut-off point was 32 pg/ml for IL-6 and 12 pg/ml for TNF-α. Both of these showed a sensitivity of 98.5% in combination and hence considered to be a highly sensitive marker of sepsis in the immediate post natal period (Silveria and Procianoy, 1999). In another study, it has been shown that cytokines released in sepsis have a crucial role in stimulating the production of Nucleated RBC (NRBC) independent of hypoxia, specifically in the patients of early onset sepsis. Hence, the increased NRBC count immediately after birth could serve as an important marker of EONS in absence of hypoxia and needs to be evaluated (Dulay *et al*., 2008).

2.6.4.6 Cell surface markers

CD64, the high affinity Fc receptor for IgG is expressed on circulating polymorphonuclear (PMN) cells and monocytes during systemic inflammatory

response and considered to be an index of host immune response to bacterial infection. Hence, CD64 is accounted as a useful diagnostic marker for neonatal sepsis, specifically in early onset sepsis, with a sensitivity and specificity of 80% and 79% respectively (Bhandari *et al*., 2008). The expression of another cell surface marker CD11b, a subunit of the b2 integrin adhesion molecule also gets upregulated on neutrophil during sepsis in neonates. The sensitivity and specificity of CD11b for diagnosing early onset sepsis are 96-100% and 81-100% respectively (Turunen *et al*., 2006). Other leukocyte markers like CD48, CD14, CD25, CD28 and CD18 have also been found to be valuable as diagnostic marker for neonatal sepsis (Pierrakos and Vincent, 2010).

2.7 Prevention

2.7.1 Before Delivery

- Improvement of maternal nutrition and proper monitoring of maternal health before delivery is directly associated with improved neonatal health and reduced infections (Darmstadt *et al*., 2005).
- Maternal immunization is also an effective way of preventing neonatal infection as it provides neonates with appropriate antibodies as soon as they are born (Healy and Baker, 2007).

2.7.2 During Delivery

- Hygienic and clean delivery practices have been evidenced in many studies to reduce rates of neonatal sepsis in both home and health facility settings (Rhee *et al*., 2008).
- In developing countries, most of the deliveries take place in home. This practice should be reduced as much as possible to lower down the incidence of sepsis in the newborns (Waters *et al*., 2011).

- Intrapartum antibiotic prophylaxis has also been found as a highly effective way to reduce both early–onset neonatal and maternal sepsis in developed countries (Ohlsson and Shah, 2009).

2.7.3 After Delivery

- Overcrowding and understaffing in NICU should be avoided (Harbarth *et al*., 1999).
- Proper hygienic practices like handwashing, use of sanitizers among the staffs and workers of NICU after delivery has been evidenced to control sepsis and infection rates in the newborns (Rhee *et al*., 2008).
- Neonatal immunization has been considered as a vital tool to lower down neonatal infection rates (Levy, 2007).
- Use of neonatal skin antisepsis preparations such as sunflower seed oil has been found to be promising (Mullany *et al*., 2006).
- Breastmilk feeding of neonates should be encouraged to prevent infection and sepsis as breastmilk contains lysozyme, secretory IgA, white blood cells and lactoferrin and has been shown to facilitate the growth of healthy Lactobacilli and reduce the growth of *E. coli* and other Gram-negative pathogens (Levy, 2007).
- Neonatal micronutrient supplementation is also considered as a preventive tool for neonatal infection. Mainly vitamin A supplementation trials are in practice (Gogia and Sachdev, 2009).

2.8 Treatment

Antimicrobial therapy

Empirical antibiotic therapy for a newborn with suspected sepsis should be unit specific and depends on several factors like the prevailing spectrum of pathogens and

the antimicrobial susceptibility patterns of the given unit. Antibiotics once started should be modified according to the culture sensitivity reports (Tripathi and Malik, 2010). For early onset sepsis penicillin or ampicillin with a combination of aminoglycosides (gentamicin) is usually advised. For community-acquired sepsis in which chances of resistant strains are less, the combination of ampicillin and gentamicin is generally suggested for first line therapy. For hospital-acquired sepsis where presence of resistant strains is more likely, a third generation cephalosporin e.g. cefotaxime, cefotriazone in combination with an aminoglycoside is given. Cefotaxime is advantageous to other third-generation cephalosporins for use in neonates as it does not displace bilirubin from albumin and does not cause hypoprothrombinemia and bleeding like ceftriaxone (Tripathi and Malik, 2010; Camacho-Gonzalez *et al*., 2013).

Reserve antibiotics

These antibiotics are not of empirical use, these are reserved for situations where sensitivity of the isolate justifies its use. Newer antibiotics such as meropenem, aztrenam, imipenem are listed in this category and are effective against a broad range of pathogens. Imepenem has excellent activity against most bacterial pathogens except methicillin resistant *Staphylococcus aureus* (MRSA) and *Enterococcus*. Aztreonam is effective against Gram-negative organisms (Tripathi and Malik, 2010).

2.9 *E. coli* in neonatal sepsis

E. coli is the second most common cause of neonatal sepsis in term infants and the most common cause in VLBW neonates with rates of 5.09 per 1000 live births (Fig. 2.4) (Stoll *et al*., 2011; Weston *et al*., 2011). The incidence of EONS caused by *E. coli* has increased as shown in recent studies and *E. coli* is now considered to be the most important agent for EONS (Stoll *et al*., 2011; Lin *et al*., 2011). In addition, cases

of poor outcomes leading to high mortality in newborns with early-onset *E. coli* sepsis is also evidenced (Mayor-Lynn *et al*., 2005).

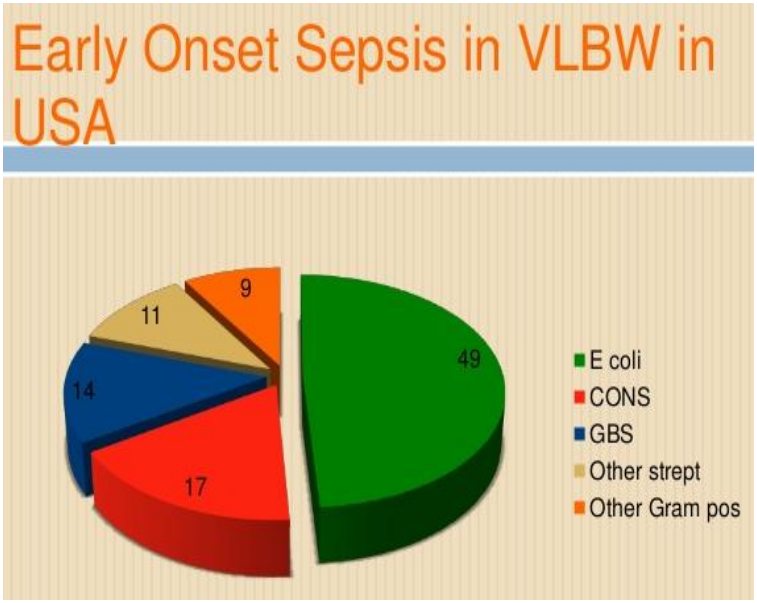

Fig. 2.4 Early onset sepsis in VLBW neonates in USA. (Stoll BJ *et al*., 2005).

In premature infants, the bacterial epidemiology has completely changed during the last 10 years with *E. coli* becoming largely predominant (Paolucci *et al*., 2012). In a study, it was found that 9 neonates were born at less than 30 weeks of gestation, and 6 of them died suggesting that *E. coli* sepsis is more fatal in premature infants (Tsai *et al*., 2012). *E. coli* is frequently associated with EONS as it can colonize mother's genital tract and infect neonates at the time of delivery. For neonatal infection to take place, *E. coli* strains have to adapt to various ecological media like:

(i) The physicochemical conditions of the vaginal cavity are very different from those of the intestinal tract

(ii) The bacteria have to cross the endocervix and survive in the amniotic fluid and

(iii) The bacteria may be subjected to strong selection pressure by these conditions before generating neonatal septicaemia (Watt *et al*., 2003).

E. coli is also the leading cause of community-acquired LOS (Louvois *et al*., 1994). *E. coli* can colonize newborns from the caregivers in the NICU and may develop infection later. Environmental sources include ventilation systems and storage shelves (Alos *et al*., 1993).

2.9.1 Clinical categories of *E. coli*

E. coli isolates of biological significance to humans can be broadly classified into 3 major groups: commensal *E. coli*, intestinal pathogenic *E. coli* (IPEC) and extraintestinal pathogenic *E. coli* (ExPEC) (Russo and Johnson, 2000).

2.9.1.1 Commensal *E. coli*

E. coli is the predominant aerobic organism in the gastrointestinal tract. Commensal *E. coli* isolates represent a major part of normal facultative intestinal flora in most humans and other mammals and birds which serenely co-exist with the host and do not ever cause disease within the host intestinal tract (Russo and Johnson, 2000). In the digestive tract, commensal *E. coli* strains are located in the large intestine, particularly in the caecum and colon. The isolates colonize the mucus layer covering the intestinal epithelial cells and are shed into the intestinal lumen with the degraded mucus components and excreted in the feces (Tenaillon *et al*., 2010). The prevalence and density of *E. coli* in the gastrointestinal tract depends on several host factors like distinct body sizes, gut morphologies, diets, digesta retention time and microbiota. In humans, the prevalence of *E. coli* in the GI tract is more than 90% but in wild mammals it is only 56%, 23% in birds and 10% in reptiles. In humans, the concentration of *E. coli* per g of feces varies from 10^7 to 10^9 CFU (Penders *et al*., 2006). *E. coli* is among the first bacterial species to colonize the intestine during infancy, reaching very high density (higher than 10^9 cfu per g of feces) before the expansion of anaerobes. The population stabilizes after 2 years and remains at around

10^8 cfu per g of feces until it gradually decreases in the older age (Tenaillon *et al*., 2010). Commensal *E. coli* isolates typically belong to phylogenetic groups A and B1 and lack most of the specialized virulence traits as possessed by the disease causing intestinal and extraintestinal *E. coli* strains. However, in certain cases like in presence of a foreign body like urinary catheter, host compromise or a high or a mix bacterial species inoculum (e.g. with fecal contamination of peritoneal cavity) commensal *E. coli* can take part in extraintestinal infections (Russo and Johnson, 2000; Russo and Johnson, 2003).

2.9.1.2 Intestinal pathogenic *E. coli* (IPEC)

In contrast to commensal strains, IPEC possesses unique VFs and cause gastroenteritis or colitis. IPEC isolates are derived from phylogenetic groups A, B1, or D, or from ungrouped lineages. Six distinct pathotypes have been described for intestinal pathogenic *E. coli* such as enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EAEC), shiga-toxin producing (STEC) and diffusely adherent (DAEC) (Kaper *et al*., 2004).

2.9.1.3 Extraintestinal Pathogenic *E. coli* (ExPEC)

ExPEC causes extraintestinal infections including UTI, pneumonia, several intra-abdominal infections, osteomyelitis, cellulitis, soft-tissue infection and meningitis. Sepsis or bacteremia can accompany infection at any of these sites. ExPECs are epidemiologically and phylogenetically different from both the commensal and the intestinal pathogenic strains. In contrary to commensal and intestinal pathogens, ExPEC derive predominantly from phylogroup B2 and to a lesser extent group D, and from specific clones within these groups, which can be recognized by their characteristic O:K:H serotypes. ExPECs do not cause gastrointestinal diseases in humans (Russo and Johnson, 2003). However, they can asymptomatically colonize

the host intestinal tract for prolonged period of time and, under certain circumstances may actually be more effective intestinal colonizers than typical fecal commensal strains. ExPEC actually initiates its association with the host primarily as a commensal and can cause disease only when they exit the gut and enter a sterile body site, particularly in situation where host is immunocompromised (Johnson and Russo, 2001). Compromised hosts with functional or anatomical abnormalities of relevant defense systems are more likely than normal hosts to become infected by *E. coli* strains of even low virulence potential (Johnson and Russo, 2001). Although certain clonal lineages with a high ExPEC virulence potential can be deduced, the inability to clearly distinguish between commensal and ExPEC isolates validates that ExPEC are facultative pathogens (Dobrindt *et al*., 2010; Tenaillon *et al*., 2010). ExPEC strains possess a set of unique VF genes (distinct from VFs of intestinal pathogens) which enable them to cause infections outside the GI tract, in both normal and compromised hosts (Johnson and Stell, 2000; Picard *et al*., 1999). These factors can also be considered to be fitness factors broadly distributed among commensals (Kohler and Dobrindt, 2011). This hypothesis is supported by early emergence of the phylogroup B2 within the species (Le Gall *et al*., 2007). Furthermore, ExPEC VFs probably evolved because of their importance for a commensal lifestyle as they can promote intestinal colonization and survival within the normal gut environment, ExPEC adhesins, siderophore systems, and toxins can be correlated with successful gut colonization in humans (Diard *et al*., 2010; Johnson *et al*., 2008).

The virulence of ExPEC is not just about the mere presence or absence of a particular VF gene, the variable expression, secretion and functional diversity of VFs are worthy of consideration (Johnson and Russo, 2001). For example, the *fim* operon which encodes type 1 fimbriae, is present in almost all *E. coli*, pathogenic or commensal, but

the level of expression is different between the two. Even, the expression is more in patients with cystitis than pyelonephritis.

2.9.2 Phylogrouping of *E. coli*

E. coli isolates are classified into four major phylogenetic groups namely 'A', 'B1', 'B2' and 'D'(Herzer *et al*., 1990; Clermont *et al*., 2000). *E. coli* isolates causing extra-intestinal infections are mainly derived from phylogroup B2 and to a lesser extent phylogroup D (Salender *et al*., 1986; Bingen *et al*., 1998; Picard *et al*., 1999; Johnson and Stell, 2000), whereas most commensal isolates belong to phylogroups A and B1 (Picard *et al*., 1999; Duriez *et al*., 2001; Das *et al*., 2013). However, there are a few exceptions in which the ExPECs comprise of phylogroups A and B1 (Abdallah *et al*., 2011). Isolates of phylogroup B2 are mostly found to carry the specialised virulence genes when compared to the isolates of other groups (Picard *et al*., 1999; Johnson and Stell, 2000).

2.9.3 Virulence factors (VFs) of septicemic *E. coli*

2.9.3.1 Iron acquisition system

Iron is an essential cofactor for bacterial metabolism. Iron in ferric state is highly insoluble, giving a free-iron concentration of 10^{-18} M at pH 7. In mammalian host, free iron concentrations are extremely low, being approximately 10^{-25} M in blood and much lower at other sites and almost all of this iron is bound to host proteins such as transferrin reducing its availability to bacterial cells (Neilands *et al*., 1985). ExPECs including septicemic *E. coli* isolates manage this scarcity of iron by producing some low molecular weight compounds termed siderophores which efficiently compete with host iron-binding proteins and sequester iron from host environment. Once bound with iron, these siderophores are taken up by bacteria by specific receptors on

bacterial membranes, hence, allowing the bacteria to thrive in low-iron conditions (Ron, 2010).

Septicemic strains encode several iron uptake systems or siderophores of which aerobactin is the most abundant and effective whose presence is correlated with virulence and is usually located on ColV plasmid (Zgur-Bertok *et al*., 1990; Valvano and Crosa, 1984; Gophna *et al*., 2003). Aerobactin is formed from the condensation of two lysine molecules and one citrate (Neilands *et al*., 1985). In almost all *E. coli* strains, the aerobactin system is encoded by a five-gene operon, four genes encode enzymes for aerobactin systhesis and the fifth gene codes for outer membrane receptor protein. Upon binding with iron, aerobactin is taken up by a 74-kDa outer membrane receptor protein (De Lorenzo and Neilands, 1986).

Another major iron uptake system is similar to that located on the HPI (high pathogenicity island) typical of pathogenic *Yersinia.* The HPI encodes for the siderophore yersiniabactin and its receptor on membrane. The presence of yersiniabactin iron uptake system is present mainly in ExPEC strains and avian pathogenic strains (Schubert *et al*., 1998; Karch *et al*., 1999; Gophna *et al*., 2001a; Janben *et al*., 2001). Most of the septicemic strains contain both the iron uptake systems, aerobactin and yersiniabactin (Mokady *et al*., 2005).

Enterobactin is another common siderophore of *E. coli* having higher affinity constant (10^{52}) for iron than does aerobactin (10^{23}) in the deprotonated state. However, affinity constant for enterobactin at neutral pH is much lower, it is less soluble and less stable than aerobactin. Enterobactin undergoes hydrolysis for release of iron, whereas aerobactin is continuously recycled without hydrolysis (De Lorenzo and Martinez, 1988; Johnson, 1991). In addition, many septicemic strains contain an additional receptor, as the salmochelin receptor IroN, which binds the enterobactin siderophore

with higher affinity (Russo *et al*., 1999, 2002). The host does not lag behind for its protection against siderophores like enterobactin. Host protein lipocalin 2 has recently been identified as a potent counter measure for battling enterobactin-mediated iron scavenging by pathogens (Fig. 2.5). Another study has shown that lipocalin 2 functions as a bacteriostatic agent by specifically binding and sequestering enterobactin (Goetz *et al*., 2002).

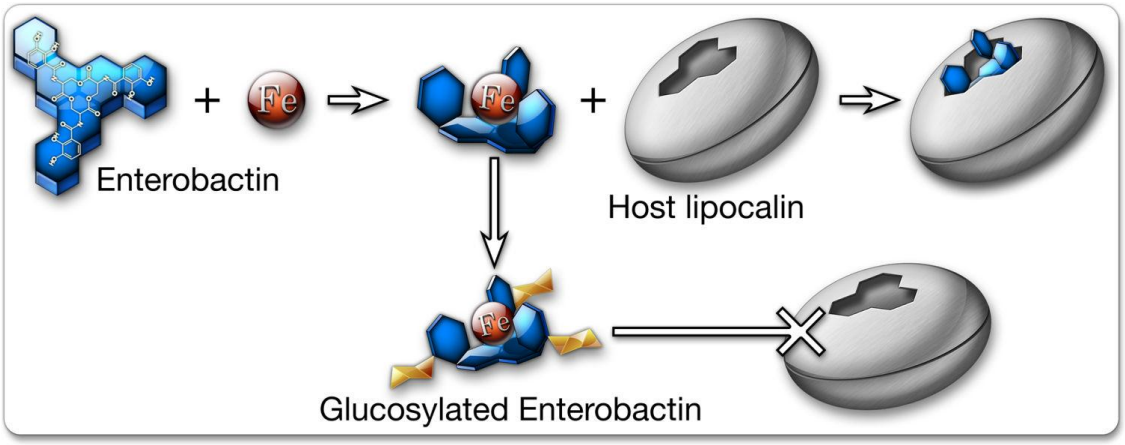

Fig. 2.5 Iron acquisition. Both host and pathogen compete for iron and have evolved multiple strategies to outdo the other. The bacterial siderophore enterobactin sequesters iron with high affinity, while host lipocalin 2 binds enterobactin and prevents its uptake by UPEC. Bacteria carrying the iroN gene cluster can modify enterobactin by glucosylation, creating salmochelin, which effectively binds iron but is not recognized by lipocalin 2 (Goetz *et al*., 2002).

2.9.3.2 Secreted Toxins

Septicemic *E. coli* secrete many toxins to damage host tissues of which α-hemolysin (HlyA) is tremendously potent and demands critical attention. HlyA is a 110 kDa calcium-dependent pore-forming toxin which belongs to the family of RTX (repeats in toxin) toxins that are widely distributed among the Gram-negative pathogens (Eberspacher *et al*., 1989; Bhakdi *et al*., 1986; Ostolaza and Goni, 1995). HlyA has been shown to have concentration dependent dual functions on primary epithelial

cells of renal proximal tubules origin (Laestadius *et al*., 2002). HlyA, at higher doses can lyse cells by inserting into membranes producing cation-selective channels of high conductance with a diameter of 2 nm, leading to lysis of erythrocytes and effector immune cells and facilitating the release of nutrients and other factors such as iron needed for bacterial growth. Lysis of diverse host cells leads to inflammation, tissue injury and impaired host defenses (Johnson, 1991; Ostolaza and Goni 1995; Keane *et al*., 1987; Cavalieri *et al*., 1984). At lower concentrations, HlyA can induce apoptosis of target host cells, including neutrophils, T lymphocytes and renal cells and promote the exfoliation of bladder epithelial cells, resulting in increased production of IL-6 and IL-8 (Chen *et al*., 2006; Smith *et al*., 2006; Russo *et al*., 2005). HlyA has also been shown to induce Ca^{2+} oscillations in renal epithelial cells, resulting in increased production of IL-6 and IL-8 (Uhle´n *et al*., 2000).

Another secreted toxin of secpticemic *E. coli* is cytotoxic necrotising factor 1 (CNF 1), a 113 kDa protein, which stimulates formation of actin stress fibers, lamellipodia, filopodia, membrane ruffle and modulation of inflammatory signaling pathways in a Rho GTPase-dependent manner (Bien *et al*., 2012). CNF1 has also been indicated in interfering with polymorphonuclear phagocytosis and triggering apoptotic death of bladder epithelial cells (Mills *et al*., 2000; Fiorentini *et al*., 1997). To exert its effects, CNF1 must gain access to the host cytosol by binding to the laminin receptor precursor on the surface of target cells, triggering uptake and subsequent trafficking of the toxin into a late endosomal compartment. Acidic pH of the compartment induce the translocation of CNF1 catalytic domain across the vesicular membrane and into the host cytosol where it stimulates Rho family GTPases. Prolonged activation of Rho GTPases leads to their ubiquitination and subsequent proteasomal degradation. So cytotoxicity associated with CNF1 is due to both aberrant Rho activation and

subsequent Rho degradation (Lemonnier *et al*., 2007). Activation of Rho GTPases affect formation of actin stress fibers, lamellipodia, filopodia, induction of membrane ruffling and modulation of inflammatory signalling pathways (Etienne-Manneville and Hall, 2002).

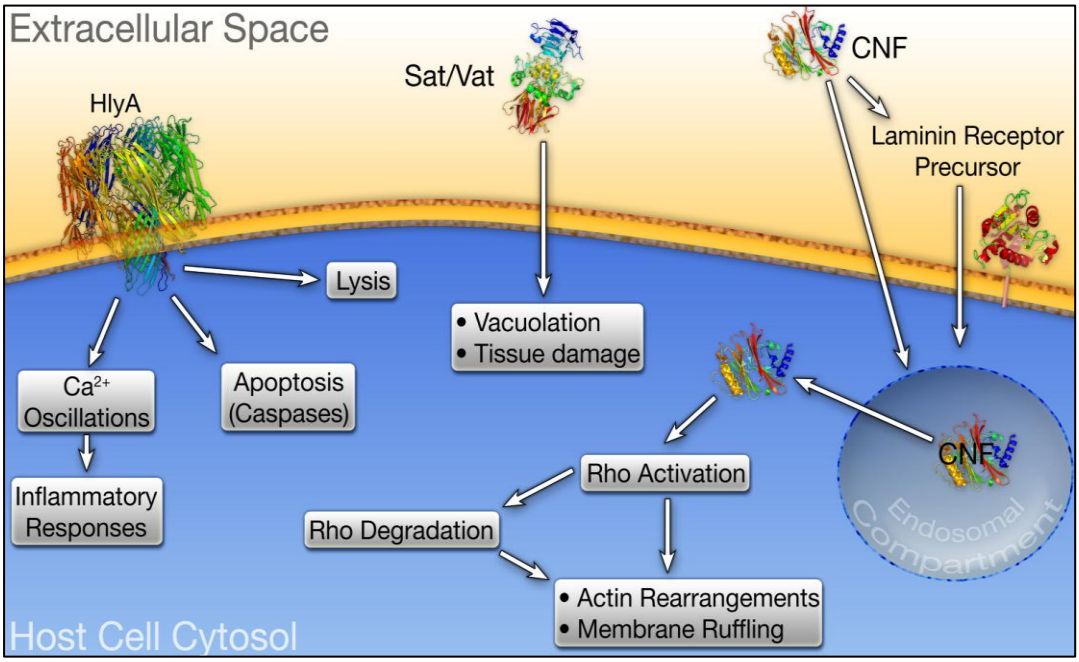

Fig. 2.6 Virulence factors in host cell invasion by ExPEC. The Fim H adhesin localized at the distal tips of type 1 pili engages α3β1 integrin receptors, and possibly other receptors, which likely cluster within cholesterol-rich lipid rafts. Receptor binding triggers signaling cascades involving FAK, Src, PI 3-kinase, Rho GTPases like Rac1, phosphoinositides (PIPs), and transient complex formation between the cytoskeleton stabilizing and scaffolding proteins α-actinin and vinculin. These events stimulate actin rearrangements, causing the host plasma membrane to zipper around and envelope bound bacteria (Wiles *et al*., 2008).

2.9.3.3 Adhesins

The adhesins of pathogenic bacteria not only promotes bacterial invasion to host tissue but can also trigger host immune response and facilitates delivery of other bacterial products to host tissues (Mulvey, 2002). The primary adherence factor or adhesin for septicemic isolates are the fimbriae or pili. The septicemic strains

elaborate type 1, P, S and F1C pili encoded by the *fim*, *pap*, *sfa* and *foc* operons, respectively (Wiles *et al*., 2008).

P pili are the most common adhesion factor for septicemic strains. P pili consist of heteropolymer fibres composed of different protein subunits, encoded by the *pap* (pyelonephritis-associated pili) A-K gene operon (Hull *et al*., 1981). PapA (19.5 kDa) is the major subunit needed for the formation of the fimbriae, not for receptor mediated interactions. PapF-PapG constitutes the minimal adhesion complex, which is linked to fimbriae by PapE. PapG (35 kDa) which is located at the distal tip of the P pilus, is actually the adhesin required for binding to the receptor. PapF is also important in the initiation of subunit polymerization (Johnson, 1991). The receptor for P fimbriae is glycosphingo lipids containing the Gal-α (1-4) Gal determinant (Wullt *et al*., 2000). The adhesion of P fimbriae to this receptor leads to the release of ceramide, which acts as an agonist of toll-like receptor 4 (TLR4), and trigger local inflammatory response (Fischer *et al*., 2007). P fimbriae are classified according to their iso-receptor specificity. Class I P fimbriae carry $PapG_{J96}$ adhesin, which binds to globotriaosylceramide (GbO3), and is uncommon in clinical isolates (Johnson *et al*., 1997). Class II P fimbriae carry $PapG_{IA2}$ adhesin and binds to most members of the globoseries of GSLs or GbO3 and recognize all P blood group determinants, have a strong association with pyelonephritis in children and adult women (Johanson *et al*., 1993; Otto *et al*., 1993). Class III G adhesion, encoded by PrsGJ96 sequences, binds to sheep erythrocytes or GbO5, recognize P blood group determinants with a terminal blood group A residue (Johanson *et al*., 1993; Lund *et al*., 1988).

The S fimbriae with its subtypes *sfa I* and *sfa II* are frequently associated with *E. coli* that cause sepsis. S fimbriae binds to epithelial and endothelial cell lines from various tissues and facilitate bacterial dissemination within the host tissues (Marre *et al*.,

1990). S fimbriae recognize specific α-sialyl-2-3-β-galactose containing receptors (Parkkinen *et al*., 1983) and mediate X-type MRHA of human erythrocytes, a property that can be exploited to separate S-fimbriated cells from a mixed population (Nowicki *et al*., 1985). The S-fimbrial adhesion is located at the fimbrial tips and as with P and type 1 fimbriae and has a different amino acid sequence than the structural subunit (Moch *et al*., 1987).

Afimbrial (Afa) adhesins are also associated with *E. coli* strains causing sepsis. This family of non-fimbrial adhesions bind to various portions of the Dr blood group antigen These adhesins differ from other *E. coli* fimbrial adhesins and appear as a fine mesh, a coli like structure or a filamentous capsular coating on the cell surface (Labigne-Roussel and Falkow, 1988; Labigne-Roussel *et al*., 1984).

2.9.3.4 Secretion systems

E. coli possess genes of the type III secretion system ETT2 (*E. coli* type III secretion system 2) to facilitate the translocation of secreted toxins into host cells (Kenny, 2002; Nougayrede *et al*., 2003). In septicemic *E. coli* strains, a unique version of the type III secretion system ($ETT2_{sepsis}$), homologous to ETT2 of strain O157 can be found (Hartleib *et al*., 2003; Ren *et al*., 2004). $ETT2_{sepsis}$ is a degenerate operon containing a large deletion, conserved in many septicemic *E. coli* strains and is essential for sepsis (Ideses *et al*., 2005). $ETT2_{sepsis}$ has evolved in such a way that it apparerntly does not function in secretion, but is involved in the septicemic survival in the host (Ron, 2010).

Interestingly, some outer membrane proteins are also secreted through these secretion systems. The secretion of OmpA by a septicemic *E. coli* O78 strain may play a role in virulence as OmpA has been shown to bind neutrophil elastase, which has bactericidal effects (Gophna *et al*., 2004).

2.9.3.5 Serum resistance

Due to the presence of complement and other inhibitory factors, serum is bactericidal. Now, several VFs of pathogenic bacteria are potentially involved in serum resistance, the molecular mechanism of which is unclear till now. The principle components for serum resistance are polysaccharide capsules and LPS. These two VFs have been of great diversity among the septicemic strains, including more than 20 LPS serotypes and a variety of capsules (Ron, 2010). The polysaccharide K1 capsule is protective against complement-mediated killing and baceriophages, therefore, enhancing bacterial survival in blood and in brain microvascular endothelial cells and evading bacterial phagocytosis by phagocytes (Scholl *et al*., 2005; Kim *et al*., 2003; Allen *et al*., 1987). In recent years, other genes like *traT* has been identified for increasing serum survival of *E*. *coli* pathogenic strains (Johnson and Stell, 2000).

2.9.3.6 Genes involved in internalization in host cells

So far the only gene identified for bacterial internalization in eukaryotic cells is the gene encoding curli fibers. These thin aggregative surface fibers bind several host molecules including laminin, fibronectin, plasminogen, human contact phase proteins and major histocompatibility complex (MHC) class I molecules. Bacteria with curli mutation showed lower level of colonization, invasion, and persistence in 1-day-old chicks (Gophna *et al*., 2001b,c, 2002).

2.10 Serine Protease Autotransporters of *Enterobacteriaceae* (SPATEs)

As the name implies, autotransporters (ATs) are a family of proteins that mediate their own secretion through the outer membrane of Gram-negative bacteria. This secretion mechanism is also known as type V secretion. These ATs are actually quite diverse in their function and can act as lipases, esterases, adhesins and proteases. SPATE, the subfamily of autotransporters is a group of secreted serine proteases of

Enterobacteriaceae (Henderson and Nataro, 2001). Provence and Curtiss in 1994 described the first SPATE protein: the temperature-sensitive-hemagglutinin (Tsh) from an avian pathogenic *E. coli* (APEC) strain (Provence and Curtiss *et al*., 1994). Since then, more than 20 SPATEs have been reported so far (Yen *et al*., 2008). SPATEs are usually among the most abundant proteins secreted by the parental strain (Dautin 2010).

2.10.1 Structure of SPATEs

The member proteases that belong to the family SPATEs, share conserved identical structural features. The secreted component and the secretion apparatus of an AT system lie in the same polypeptide, which comprises of three domains. The N-terminal signal sequence, an internal passenger domain and a C-terminal translocator domain (Fig. 2.7).

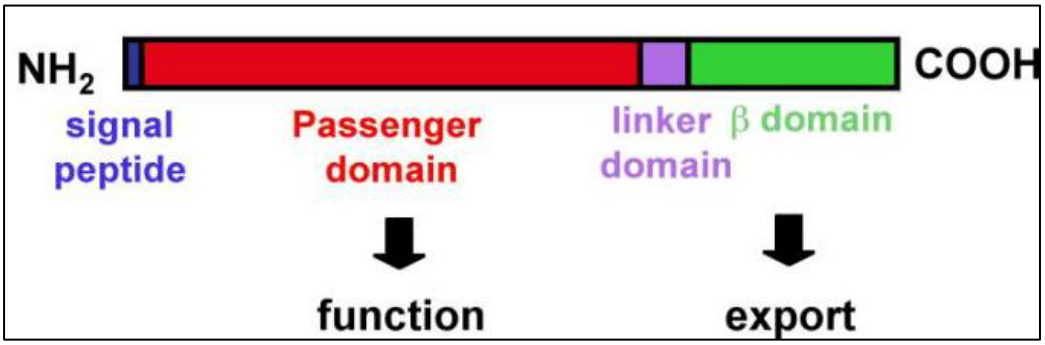

Fig.2.7 Structure and organization of SPATEs (Dautin 2010).

The N-terminal, *sec*-dependent, signal peptide is needed for targeting to- and export through the inner membrane. The C-terminal domain or the translocator domain forms a β-barrel structure in the outer membrane through which the passenger domain is translocated to the cell surface (Dautin and Bernstein, 2007b; Henderson *et al*., 1998). The passenger domain is the functional domain which consists of an N-terminal globular domain harbouring a characteristic GDSGSP serine protease motif. The first

serine of this motif forms a catalytic triad with a histidine and asparagine residue forming a substrate binding pocket. The C-terminal of the passenger domain folds into a β-helical stalk and is thought to confer structural stability to the secreted protein. The passenger domains are variable in length (between 954 and 1050 residues). In contrast to the translocator or β-domains which are 60 to 99% identical among the members of SPATEs, passenger domains are only 23 to 50% similar by their amino acids. Some SPATEs are more conserved like Tsh and Hbp which differ by only two amino acid residues. Moreover, Tsh/Hbp is 77.5% identical with Vat and SepA and 72.8% with EatA (Dautin, 2010).

2.10.2 Classification of SPATEs

Multiple sequence alignment of all the SPATEs generates different phylogenetic clusters of SPATEs. Phylogenetically, SPATEs are classified into two groups, class I and class II based on the presence or absence of a domain in the globular N-terminus of the passenger domain called domain 2. Class I SPATEs lack this domain 2 and are cytotoxic, whereas Class II SPATEs possess domain 2 and are cytopathic having various intra and extracellular proteolytic targets. However, in a recent study by Ruiz *et al*., a subset of Class II SPATEs without domain 2 has been identified, making this no longer the distinguishing feature between these two classes. Instead recent studies have identified two additional smaller domains in the globular domain, designated as domain 3 and 4 (Fig. 2.8). Domain 3 possess a pair of cysteine residues in case of Class I SPATEs, but not in Class II (Ruiz-Perez, F *et al*., 2014). Members of Class I SPATEs include Pet (plasmid encoded toxin), Sat (secreted autotransporter toxin), SigA, EspP (extracellular serine protease plasmid (p0157)-encoded), EspC (EPEC secreted protein C) and Vat (vacuolating autotransporter toxin) from *E. coli* and *Shigella flexneri*. Class II SPATEs comprises of Pic (protease involved in intestinal

colonization), SepA (*Shigella* extracellular protein A), EatA (ETEC autotransporter A) and Tsh (temperature-sensitive hemagglutinin) which has also been named as Hbp (Hemoglobin protease) (Dutta *et al*., 2002; Boisen *et al*., 2009; Dautin, 2010).

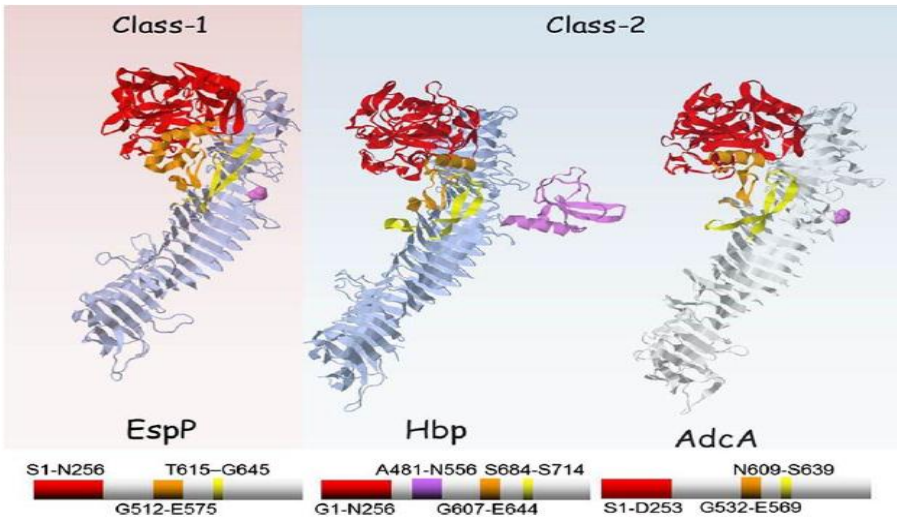

Fig. 2.8 Structure of Class I and Class II SPATE passenger domain. Proteolytic domain 1 is depicted in red, domain 2 in pink, domain 3 in orange and domain 4 in yellow. Class I SPATEs contain two cysteine residues in domain 3, forming a disulphide bond. These are absent in Class II SPATEs. C-terminal β-helical stalk is shown in blue for EspP and Hbp and in white for AdcA. (Ruiz-Perez and Nataro, 2014).

2.10.3 Mechanism of secretion

SPATEs belong to a larger family of ATs that exist in monomeric and trimeric forms. All ATs share the same domain architecture and similar mechanism of secretion. However, SPATEs possess certain features which are more conserved within the family such as presence of an extended signal peptide (50 amino acids) in contrast to other ATs which have a classical signal sequence of 25 amino acids. This classical signal sequence is recognized by signal recognition particle (SRP) which directs the polypeptide to the Sec-translocon by co-translational translocation, whereas, it is hypothesized that the additional N-terminal extension peptide prevents SRP binding to the SPATE polypeptide and possibly binds to an unknown cytoplasmic factor for

keeping SPATE in a translocation competent state for post translational translocation. Another hypothesis suggests that this N-terminal elongated signal peptide leads the SPATE polypeptide to the Sec-translocon after it destines to the periplasmic space, hence delaying signal cleavage and release into the periplasm, thereby ultimately preventing misfolding of the PD in the periplasm (Fig. 2.9). So, after synthesis SPATEs are targeted to the Sec-translocon which catalyzes their transport through the inner membrane (Dautin, 2010).

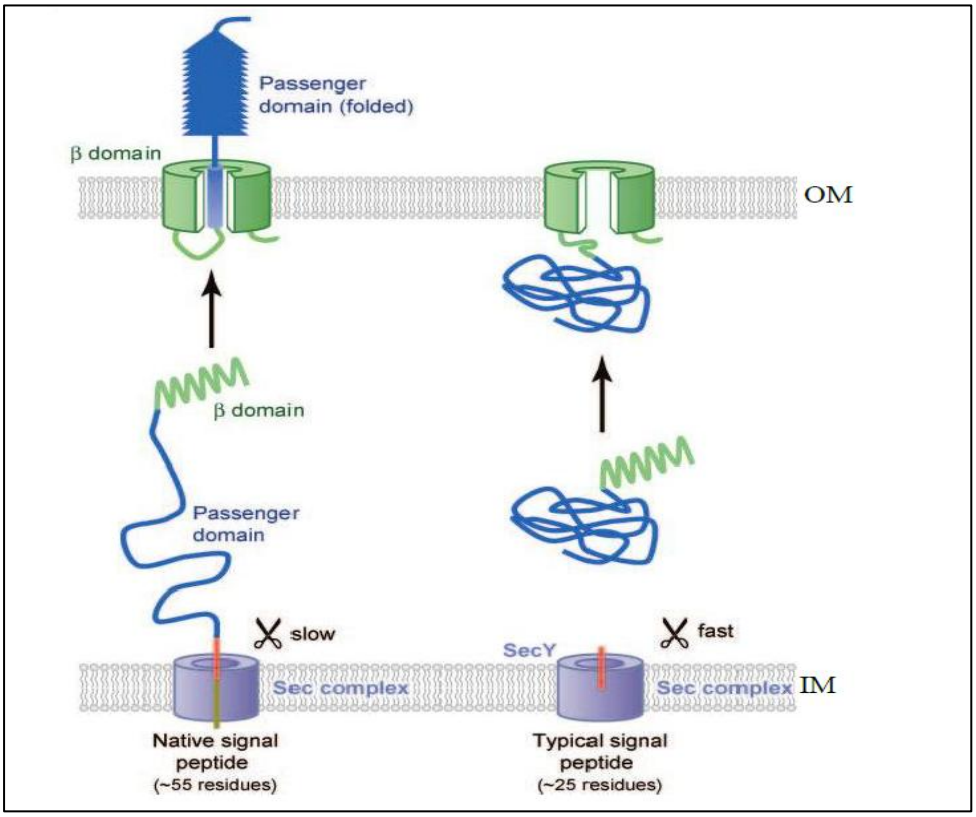

Fig. 2.9 Model for unusual SPATEs extended signal sequence. Unusual autotransporters signal peptides dissociate from the Sec complex and become accessible to cleavage by leader peptidase relatively slowly (left). This prevents it from folding into a translocation incompetent form. When native signal peptide is replaced with typical signal peptide, the PD is released from the IM more quickly and misfolds (right).

Periplasmic chaperons have a role in preventing misfolding of the PD in the periplasm. Two chaperons are reported so far, SurA and DegP. Once in the periplasm,

the β domain is inserted into the outer membrane and forms a β-barrel through which the passenger domain is transported to the extracellular medium (Ruiz-Perez *et al*., 2009).

There are certain models to explain the insertion of OM barrel and translocation of the passenger domain through the OM. In the hairpin model, the PD is transported through the pore of the β-barrel in an unfolded state (Dautin and Bernstein, 2007b). In a study of IgA protease from *Neisseria*, it has been proposed that this AT forms multimers in the OM and PD is translocated through a central channel formed by the walls of 6 or more β-barrels (Veiga *et al*., 2002). A well known model demonstrates that the Bam complex (aka YaeT/Omp85) promotes the insertion of β-barrel into the OM and the PD translocation is concomitant to the insertion of AT in the OM (Bernstein, 2007; Genevrois *et al*., 2003). Whereas a number of ATs remain intact on the surface of outer membrane after translocation, the SPATEs are autoproteolytically cleaved at an FxxEVNNLNK motif in the linker domain that connects the passenger and C-terminal translocator or β domain after the first asparagine. The cleavage occurs between two asparagine residues (Fink *et al*., 2001; Dautin *et al*., 2007a). Single amino acid mutations of the principle catalytic residues conserved among SPATEs lead to the discovery of aspartate 1120 present inside the β-barrel cavity as a key for proteolytic cleavage. Asp1120 close to Asn1023 makes a nucleophilic attack on the Asn1023-Asn1024 peptide bond leading to cyclization of Asn1023 which is ultimately hydrolyzed to Asparagine or iso-asparagine (Dautin *et al*., 2007a).

2.10.4 Role of SPATEs in pathogenesis

Even though some recent studies have also detected SPATEs in non-pathogenic strains (Sandt and Hill, 2000), SPATEs are strongly associated with pathogenic strains (Parham *et al*., 2005; Boisen *et al*., 2009). Though SPATEs share common structural features, they have distinct substrate specificities and diverse mode of

action (Dautin, 2010).

2.10.4.1 Class I SPATEs

2.10.4.1.1 EspP

EspP (Extracellular serine protease-plasmid encoded) is secreted by several serogroups of EHEC, STEC and atypical EPEC strains (Brunder *et al*., 1997; Djafari *et al*., 1997). EspP is known to cleave human coagulation factor V, an important factor in blood clotting (Brunder *et al*., 1997). EspP has also been shown to disrupt tight junction integrity in Vero cells. EspP has been found to induce cytoskeletal damage, with loss of stress fiber, cell detachment and rounding, disruption of actin cytoskeleton and opening of cell-cell junction (Djafari *et al*., 1997). Another presumed role of EspP is in the biofilm formation and acting as a substratum for bacterial adherence (Xicohtencatl-Cortes *et al*., 2010). EspP deletion significantly reduced the colonization levels in EHEC.

2.10.4.1.2 EspC

EspC (EPEC secreted protein C) is chromosomally located and produced by EPEC (Mellies *et al*., 2001). Purified EspC was found to exhibit enterotoxicity on rat jejunal tissue mounted in Ussing chambers (Mellies *et al*., 2001) as well as cytotoxicity on epithelial cells *in vitro (*Navarro-Garcia *et al*., 2004). Though EspC was able to bind to fodrin *in vitro*, it did not cause fodrin redistribution into aggregates in the damaged cells. This was attributed to the differential proteolytic specificity of EspC to fodrin cleavage, i.e. EspC cleaved fodrin at two sites both outside of the calmodulin-binding domain of fodrin *(*Navarro-Garcia *et al*., 2004). Mode of entry of EspC into the epithelial cells is still not clear. When EPEC rather than just purified EspC was applied to HEp-2 cells, EspC internalization was enhanced within 3 hrs of infection, indicating the involvement of a bacterial factor in this toxin delivery (Vidal and Navarro-Garcia, 2006). The role of type III secretion system is also hypothesized for

delivery of EspC. EspC after secretion through type V secretion system, interacts with components of EPEC Type III secretion system (EspA, the tip protein) and is internalized by a process dependent upon this system. Protein overlay experiments showed direct interaction between EspC and EspA, hence showing crosstalk between the two secretion systems for the first time (Vidal and Navarro-Garcia, 2008).

2.10.4.1.3 Pet

Pet (Plasmid-encoded toxin) is associated with EAEC (Eslava *et al*., 1998). Pet was the first AT in which both enterotoxic and cytotoxic activity was reported. Mucosal toxicity and exfoliation of intestinal cells in EAEC induced diarrhea is attributed to Pet (Henderson *et al*., 1999). Pet shows enterotoxic activity on rat jejunal tissue mounted in Ussing chambers (Navarro-Garcia *et al*., 1998). It also exhibits cytotoxicity on HEp-2 and HT-29 cells (Fig. 2.10). In cytosol, Pet cleaves fodrin like EspC and hence tempts redistribution of fodrin. P*et al*so induces redistribution of focal adhesion complex through cleavage of FAK (focal adhesion kinase), an early phenomenon that occurs along with fodrin cleavage. These ultimately lead to cytoskeletal disruption and cell exfoliation (Cappello *et al*., 2011).

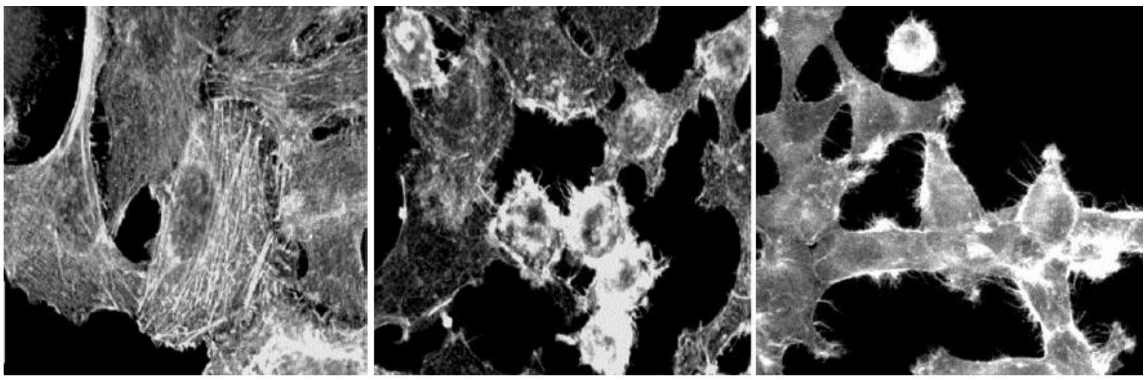

Fig. 2.10 Cytotoxicity of EspC and Pet on HEp-2 cells. Untreated HEp-2 cells (left), Treatment with EspC at 120 µg/ml for 8 hrs (middle), treatment with Pet 40 µg/ml for 4 hrs (right) (Nataro *et al*., 2004).

2.10.4.1.4 SigA

SigA (*Shigella* IgA1-protease like) is secreted by *Shigella flexneri*. SigA was discovered in the *she* pathogenicity island of *S. flexneri*, along with other SPATE, Pic and an Ag43 like AT (AL-Hasani *et al*., 2001). SigA was shown to cause cell rounding and detachment of HEp-2 cells, fodrin redistribution and cleavage of fodrin at the calmodulin-binding domain (AL-Hasani *et al*., 2009). Wild type *S. flexneri* caused more fluid accumulation in rabbit ileal loop compared with the SigA mutant (AL-Hasani *et al*., 2000). In contrast with EspC and Pet, the mechanism of SigA entry in target cells and trafficking route following entry has not been determined. High anti-SigA titers were detected in infected individuals (AL-Hasani *et al*., 2009) and also in patients with inflammatory bowel disease. Hence, SigA is a putative candidate for developing vaccines for *Shigella*-induced dysentery (De Souza *et al*., 2012).

2.10.4.1.5 Sat

Sat (Secreted autotransporter toxin) was identified in CFT073, an uropathogenic *E. coli* isolate isolated from blood and urine of a woman with acute pyelonephritis. Though present in fecal isolates, Sat is predominantly expressed in pyelonephritis-associated UPEC strains and UTI-associated Afa/Dr DAEC strains (De Souza *et al*., 2012). Several studies have shown the prevalence of Sat among strains of UPEC than in commensal or diarrheagenic *E. coli* strains (Restieri *et al*., 2007). Sat is cytotoxic on Vero kidney cells, HK-2 human bladder and HEp-2 cell lines, and promotes cells elongation and detachment from their support (Guyer *et al*., 2000). Sat also causes cytoplasmic vacuolation of kidney and bladder epithelial cells (Guyer *et al*., 2002). Sat is also able to cleave fodrin in vitro. The contribution of Sat in DAEC pathogenesis has also been reported. Sat was responsible for lesion in tight junctional domain of polarized Caco-2/TC7 monolayers. Sat modified tight junction proteins

ZO-1, ZO-3, occludin and claudin and increased para-cellular permeability in the mucosal to serosal direction (Guignot *et al*., 2007).

2.10.4.2 Class II SPATEs

2.10.4.2.1 Vat

Vat (vacuolating autotransporter toxin) is encoded on Vat pathogenicity island (Vat-PAI) adjacent to the *thrW* tRNA gene in avian pathogenic *E. coli* (APEC) and causes extraintestinal diseases like cellulitis, septicaemia and respiratory diseases in poultry. The presence of Vat at this position has been demonstrated for UPEC strain CFT073 and neonatal meningitis strain *E. coli* RS218. In a work with APEC, Vat caused cytoplasmic vacuolation of chicken embryo fibroblasts and primary chicken kidney cells in a manner similar to VacA of *H. pylori*. No vacuolating activity could be reported for Tsh despite being 75% identical to Vat. Interestingly, Vat is the only SPATE reported so far for which the active site catalytic sequence GDSGSP is altered by ATSGSP. No substrates have been identified for Vat so far (Parreira and Gyles, 2003).

2.10.4.2.2 Tsh

Tsh (temperature sensitive hemagglutinin) is the first SPATE to be described and it was identified in an APEC strain (Provence and Curtiss, 1992; 1994).Tsh was found to promote temperature sensitive hemagglutination of chicken erythrocytes, being most active at 26ºC, less active at 37ºC and inactive at 42ºC (Provence and Curtiss, 1992). Conjugation and hybridization experiments revealed that *tsh* gene is located on a ColV-type plasmid, near the colicin V genes in many of the APEC strains. *tsh* was found to possess homology with four serologically distinct *H. influenzae* genes and *N. gonorrhoeae* IgA1 protease (Provence and Curtiss, 1994). Binding studies of Tsh with extracellular matrix proteins showed that Tsh binds with fibronectin, collagen IV and

mucin in a dose dependent manner independently of the catalytic serine residue. Like Hbp, Tsh can degrade and bind hemoglobin (Kostakioti and Stathopoulos, 2004). Tsh is also expressed by UPEC but the role in UTI is still unclear (Heimer *et al*., 2004).

2.10.4.2.3 Hbp

Hbp (hemoglobin protease or hemoglobin binding protein) was initially discovered in an *E. coli* strain EB1 isolated from a patient with wound infection. Hbp can bind both heme and hemoglobin *in vitro* with a better affinity for hemoglobin than heme. Hbp can also degrade hemoglobin (Otto *et al*., 1998). Hbp and Tsh are 99.9% identical, differ only by two amino acid residues. However, Hbp in contrast with Tsh, does not show any mannose-resistant hemagglutination activity (Otto *et al*., 1998). Like Tsh, Hbp is able to induce abcess formation in a mouse model of coinfection with *B. fragilis*. These suggest that the two diverse amino acid residues of Hbp and Tsh are responsible for heme/ hemoglobin binding as well as the hemagglutinating activity but not for abcess formation (Otto *et al*., 2002).

2.10.4.2.4 Pic

Pic (protein involved in colonization) was identified in EAEC (Henderson *et al*., 1999) and *Shigella flexneri* (Rajakumar *et al*., 1997). In contrast with Pet, Pic was identified in EAEC chromosome. Later, a homologue of Pic was also identified UPEC, termed PicU which shared 96% homology with Pic (Heimer *et al*., 2004; Parham *et al*., 2004). Subsequently it was revealed that PicU was expressed during experimental infection in the mouse model of UTI (Heimer *et al*., 2004). The flanking sequences of Pic in EAEC and *S. flexneri* are not the same, in case of *S. flexneri*, *pic* gene is flanked with another SPATE, *sigA* on the *she* PAI. Pic was shown to confer serum resistance, the exact mechanism of which is not clear but probably Pic degrades one of the components of the complement classical pathway of activation (Henderson

et al., 1999). No cytotoxicity of Pic could be detected (Navarro-Garcia *et al*., 1998). However, it was shown that Pic can cleave fodrin (Parham *et al*., 2004). It has been shown that Pic has mucinase activity, which was elicited in an *in vivo* rat ileal loop infection model. Wild type EAEC, UPEC, *S. flexneri* but not the *pic* mutants showed hypersecretion of mucin into the ileal lumen (Navarro-Garcia *et al*., 2010). The mucinase activity of Pic was further evidenced by its ability to cleave CD43, CD45, CD44 and PDGL1 expressed on hematopoietic cells (Ruiz-Perez, *et al*., 2011).

2.10.4.2.5 SepA

SepA (*Shigella* extracellular protein) was initially identified in *S. flexneri*, but strains of EAEC were also found to secrete SepA. *sepA* mutant of *Shigella* was not found with altered cellular invasion or intercellular mobility. However, *sepA* mutant was unable to cause fluid accumulation in rabbit ileal loop (Benjelloun-Touimi *et al*., 1995). SepA has 72.8% homology with EatA, an enterotoxin produced by ETEC (Dautin, 2010). Substrate specificity of SepA *in vitro* showed similarity with cathepsin G. However, in contrast to cathepsin G, SepA was not able to activate platelets, cleave thrombin receptors, fibronectin, collagen or angiotensin I (Benjelloun-Touimi *et al*., 1998). Though SepA is widely distributed among pathogenic strains (Boisen *et al*., 2009; Rasko *et al*., 2011) *in vivo* target of SepA is yet to be determined.

2.10.4.2.6 EatA

EatA (ETEC autotransporter A), a plasmid encoded SPATE is strongly associated with ETEC strains. EatA has 73% similarity with SepA. It displays similar enterotoxicity in rabbit ileal loops like SepA. At 7 hrs of infection, ETEC *eatA* mutant caused less fluid accumulation and mucosal damage compared with the wild type strain. However, no difference was observed between wild type and mutant following

16 hrs of infection, indicating that EatA is not essential for ETEC virulence, but it may accelerate the development of the disease (Patel *et al*., 2004; Benjelloun-Touimi *et al*., 1995). The substrates cleaved by EatA are similar with cathepsin G and SepA (Benjelloun-Touimi *et al*., 1998).

2.11 *E. coli* metalloprotease YghJ

YghJ belongs to a large and diverse family of eukaryotic and prokaryotic proteins containing putative metalloprotease domain. This novel domain named M60-like pfam 13402 was identified in a proteomics analysis and characterized as a new sub-family of extracellular zinc (Zn)-metallopeptidases that are conserved amongst a range of host-associated bacterial and eukaryotic microbes including mutualists and pathogens of invertebrates and vertebrates. Lateral gene transfers between distantly related microbes explained their shared M60-like/PF13402 domain. Most of the M60-like/PF13402-containing proteins were predicted to possess a signal peptide, one or more transmembrane domains or a bacterial lipoprotein motif, suggesting that these proteins are either secreted or anchored at the surface of microbial cells and hence can act on extracellular targets. Different biochemical analyses have led to the hypothesis that M60-like/PF13402 containing proteins possess a canonical HEXXH motif within the M60 domain and play important roles in colonization of the invertebrate digestive tract and vertebrate mucosal surfaces by a broad diversity of commensal and pathogenic microbes (Nakjang *et al*., 2012). Interestingly, enhancin, the prototype molecule of this family most closely related to YghJ, was isolated from an insect virus and targets intestinal mucins. YghJ has also very recently been identified to be secrerted from Enterotoxigenic *E. coli* (ETEC) and has mucinase activity. This study by Fleckenstein *et al*., has shown that YghJ facilitates intestinal colonization of ETEC by degrading the major mucins in the small intestine, MUC2 and MUC3. In addition,

this study demonstrated that genes encoding YghJ and its cognate type II secretion system (T2SS), which also secretes LT, are highly conserved in ETEC and exist in other enteric pathogens, including other diarrheagenic *E. coli* and *V. cholerae*, indicating that this mucinase may represent a shared virulence feature of these important pathogens (Luo *et al*., 2014). YghJ has been found to have significant homology with *V. cholerae* colonization factor AcfD. AcfD also bears the enhancing-like M60 protease domain which suggests that AcfD is a mucinase. YghJ predominantly differs from other reported mucinases like Pic of *S. flexneri* and EAEC in both structure and function, i.e. specificity towards a particular type of mucin (Luo *et al*., 2014).

In another recent study by Fleckenstein *et al*., the chromosomally located *yghJ* gene has been documented to be secreted by 89% of the ETEC isolates, distributed over a diverse phylogenetic lineage and shown to be expressed during the course of infection of ETEC. This study suggests that YghJ is one of such proteins that trigger immune response to ETEC infection. YghJ along with EtpA (an extracellular adhesion), EatA (a mucin-degrading serine protease) and EaeH (an adhesin) have been identified as protective antigens shared by many ETEC strains and hence, demand critical attention for the development of the next iteration of ETEC vaccines (Roy *et al*., 2010).

Interestingly, in a study by Serino *et al*., YghJ was identified as one of the potential vaccine candidates for ExPEC isolates by a "substractive reverse vaccinology" approach and was shown to be the most protective antigen that provided nearly complete protection from bacteremia and mortality in a mouse model of sepsis. The distribution of YghJ was also studied among a diverse variety of *E. coli* pathotypes from human and animal origin and also among commensal isolates. Surprisingly, YghJ was found to be distributed among all the strains including commensal *E. coli*

isolates. However, the incidence was higher in case of intestinal and extraintestinal pathogens compared to the commensals. Furthermore, the analysis of expression and secretion of YghJ revealed that YghJ is expressed but not secreted among the commensal isolates (Moriela *et al*., 2010).