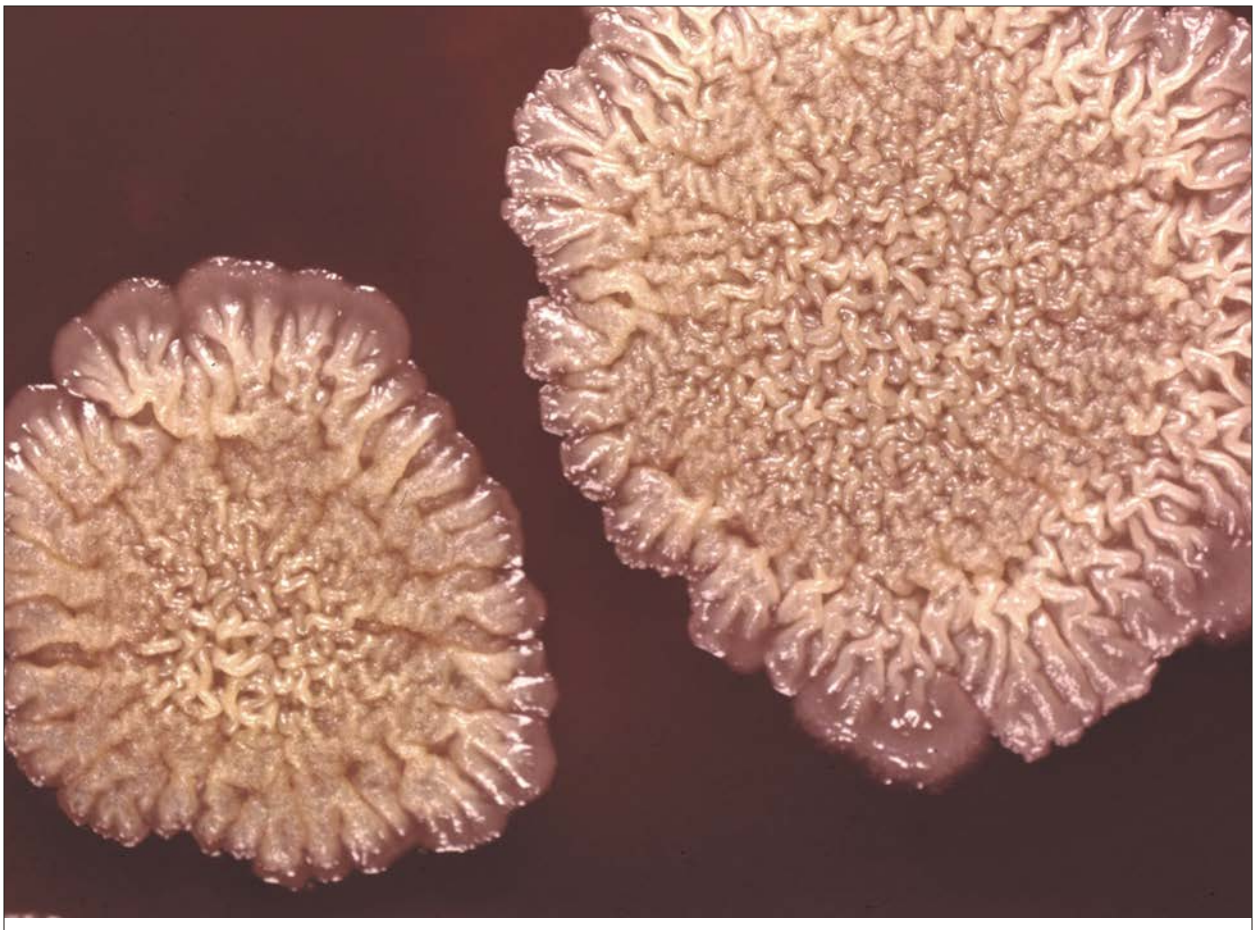

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Frequency and Characteristics of the Neonatal Sepsis Infections Caused by Extended-Spectrum Beta-Lactamase (ESBL) Producing and Non-Producing Organisms in the Chittagong Area of Bangladesh

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ABSTRACT

A total of 1365 neonates with sepsis get admitted in the Chattagram Maa-O-Shishu Medical College Hospital during the surveillance period. Among them 8.35% (114) found bacteria borne neonatal sepsis with 98.25% Gram negative and 1.75% Gram positive bacteria. *Klebsiella pneumoniae* accounted for 69.30% (79), *Serratia marcescens* 16.67% (19), *Acinetobacter* 7.02% (8), *Pseudomonas aeruginosa* 3.51% (4), *Proteus mirabilis* 1.75% (2) and *Staphylococcus aureus* accounted for 1.75% (2). Most of the Gram negative bacteria showing resistance to commonly used antibiotics (Ampicillin, Ceftriaxone and Gentamycin) but sensitivity to the Imipenem. *Klebsiella pneumoniae* is the predominant pathogen in this study, resulting in further study like ESBL and molecular pattern of resistance of the isolated *Klebsiella pneumoniae*. Present Study found that as a whole 85% (67) of *Klebsiella pneumoniae* were multi-drug resistant out of 79 strains. The 86% (68) *Klebsiella pneumoniae* detected as ESBL producer, whereas, 14% (11) revealed ESBL non producer out of 79 isolates. This study only detected the occurrence of plasmid in *Klebsiella pneumonia*. The bands were failed to separate intensely on the gel due to very similar size of plasmids between them. Pulsed Field Gel Electrophoresis (PFGE) was done to determine the relationship among the different strains of *Klebsiella pneumoniae*. Three isolated strains were analyzed to perceive the clonal relation between the strains. All the three strains gave the same patterns of band on PFGE. That means all the three strains have similar type of DNA sequence. It indicates that, these three strains causing neonatal sepsis infection may be originated from the same clone.

Key words: Neonatal sepsis, multi-drug resistance, ESBL, PFGE, *Klebsiella pneumonia*

INTRODUCTION

Over 10 million children, aged less than one year, die globally each year before their first birthday and about 40% of these deaths take place within the first 28 days of life, i.e., the neonatal

period (Ahmad *et al.*, 2000). Neonatal mortality rates vary from 5 on developed countries to 34 per 1,000 live births in the less-developed regions of the world (Ahmed *et al.*, 2002). The 98% of these deaths take place in developing countries, principally in Asia and Africa. Most births and neonatal deaths in developing countries occur in home, unseen by health care professionals (Ahmed *et al.*, 2002; Moreno *et al.*, 1994). Neonatal bacterial sepsis is one of the major cause of morbidity and mortality of Bangladeshi neonates. In developing countries, 63% of deliveries take place at home. In Bangladesh, this proportion is about 94% (Ahmed *et al.*, 1998). In Bangladesh, very few studies have been reported the causes of early and late neonatal deaths in the community (Bhatia, 1989; Bhuiya and Streatfield, 1992) and there is a death of recent published data on this issue. But Bangladesh has a neonatal death rate that is substantially high and demands urgent attention. This retrospective study was performed to determine the incidence of bacterial neonatal sepsis condition in the Chittagong area of Bangladesh with focus on various demographic characteristics of neonates like as sex, gestational age, birth weight and other factors related to neonatal sepsis (Birth place, maternal features, clinical characteristics) of neonates get admitted at Chattagram Maa-O-Shishu Medical College Hospital during the surveillance period from April, 2007 to May, 2008.

MATERIALS AND METHODS

Data collection: During the surveillance period a total of 1365 neonates were get admitted with sepsis like infection at Chattagram Maa-O-Shishu Medical College Hospital.

Blood collection: The most prominent vein was chosen to withdraw blood from infants. In very young infants, other sites were used to obtain blood specimens, such as veins in the legs or scalp.

Isolation: The 3 mL of blood were cultured in Trypticase Soy Broth (TSB) with growth supplement 1% ISO Vitale X. Normal bactericidal properties of blood and potential antimicrobial agents were neutralized by adding 0.025% sodium polyanetholesulfonate (SPS) to the culture media. Sub-culture was performed after 14-17 h of incubation, at 48 h and at day 7. The 50 μL of blood culture were inoculated on chocolate, blood and MacConkey agar.

Identification: Primary identification is based on selectivity of media, change of media after growth, morphological characteristics of the colonies and gram stain. Most of the clinically isolated Gram negative bacteria show biochemical differences on their metabolism, three sets of biochemical tests (KIA, MIU and Citrate) were performed to differentiate the isolated Gram negative bacteria from other Gram negative bacteria as well as well as to ensure the species. Single colony was picked and then stabbed into KIA followed by streaking, after inoculation at KIA; needle was directly stabbed into MIU containing indole paper. Finally, the same needle was streaked on citrate media.

Analytical profile index: The Analytical Profile Index (API® 20E strip) score was used to determine the biochemical properties of the organism. The centre of a healthy-isolated colony were carefully picked and thoroughly mixed with saline (5.0 mL) to obtain a homogenous suspension. After inoculation, ADH, LDC, ODC, H_2S and URE micro-tubes were sealed by mineral oil and then the plastic lid was fixed on tray. The strip was incubated for 18-24 h at 35-37°C in a non-CO_2 incubator.

Multi Drug Resistant (MDR) organisms: A total of 16 antimicrobials were used for this study. According to CLSI guideline resistant to three or more than three drugs designated as multidrug resistant organism. Combined resistance against Ceftazidime and Ceftriaxone also designated as multidrug resistant organism. Antibiotic sensitivity data from the antibiogram was used to detect the MDR organisms.

ESBL: Among the all isolated species *Klebsiella pneumoniae* is the predominant pathogen in this study. Resulting further study like ESBL and molecular pattern of resistance only concentrated to *Klebsiella pneumoniae*. Double Disc Synergy (DDS) test was used to determine ESBL of *Klebsiella pneumoniae*, described by Grogan *et al.* (1998) double disk ampicillin+clavulanic acid (20+10, respectively 2:1) (Oxoid) and third generation cephalosporin ceftazidime (Stoll, 1997) were used to determine ESBL. Third generation cephalosporin ceftriaxone (Stoll, 1997) was also used to confirm the ESBL producer *Klebsiella pneumoniae*.

Plasmid DNA isolation: Plasmid DNA was extracted form bacteria by high pure plasmid isolation kit (Roche, Cat No. 1754777), where cell was lysed by alkali and RNA was removed by RNase enzyme. Cellular debris and entrapped genomic DNA were removed by centrifugation; the remaining supernatant was mixed with a chaotropic salt and applied to the glass fibre fleece in a high pure spin filter tube. Under the buffer conditions used in the procedure, the plasmid binds to the glass fleece in the high pure tube while contaminating substances (salts, proteins and other cellular contaminants) remained unbind. Brief wash-and-spin steps were done to remove all contaminants. Once purified, the plasmid was easily eluted in a small volume of low salt buffer.

Pulsed-field gel electrophoresis: PFGE was performed with the Contour Clamped Homogenous Electric Field (CHEF-DRIII) apparatus form the Bio-Red Laboratories (USA). The electrophoresis chamber was filled with approximately 2.2 L of running buffer (0.5x TBE). Preparations of the electrophoresis apparatus were performed according to the instruction manual (Bio-Red, USA). The gel and the platform assembly were placed into frame. It was insured that the gel was covered by about 2 mm of buffer. The temperature of the running buffer was adjusted to 14°C and the flow rate of the buffer through the electrophoresis cell was maintained approximately at 0.75 L min^{-1}. Electrophoresis was done at 6 V for 20 h (initial switch time 2.2 sec; final switch time 63.8 sec). After the end of run, the gel was stained with ethidium bromide (0.5 μg mL^{-1}) solution for 30 min at room temperature and then de-stained in sufficient distilled water for 1 h. The gel was documented by UVP Gel-DOC. The DNA size standard was the *Salmonella* serotype *Braenderup* (H9812) ranging from 20.5 to 1, 135 kb.

Restriction digestion with XbaI: Plugs were removed from tubes containing TE with wide end of spatula and were placed in a sterile disposable petri dish and were cut at a 2 mm wide slice form test samples and transferred to the 1.5 mL micro-centrifuge tubes containing 200 μL diluted H buffer (1:10 dilution). The rest of plugs were replaced in original tubes that contained 5 mL TE Buffer. Three 2 mm wide slices of the Lambda DNA standard plug were cut and transferred to tubes of diluted H buffer. The tubes were incubated in a 37°C water bath for 10-15 min. After incubation of plug slices H buffer were removed. Then each of the plugs was immersed in 200 μL of reaction mixer which was prepared according to the calculations, described in the box. The samples and the control tubes were incubated at 37°C water bath for at least 2 h.

RESULTS

Patient enrolment: A total of 1365 neonates with suspect sepsis were enrolled in the Chattagram Maa-O-Shishu Medical College Hospital during the surveillance period. Blood culture reports were positive in 114 cases (8.35%) (Fig. 1).

Birth place of the infected child: Among the infected child 42.98% (n = 49) were born in hospital and 57.02% (n = 65) were born in home (Fig. 2).

Types of neonatal sepsis: Among the all positive cases 64.04% (n = 73) were identified as early onset sepsis and 35.96% (n = 41) were determined as late onset sepsis (Fig. 3).

Identification of the isolated organism

Preliminary identification: Gram stain provides the results that among all the isolated species 112 (98.25%) were attributing to Gram negative bacteria and 02 (1.75%) were attribute to Gram positive bacteria (Table 1).

Cultural characteristics: The isolated organism has shown typical characteristics of growth on MacConkey agar and blood agar medium. Growth on MacConkey also specified that among the six types of isolated organism five are Gram negative and one is Gram positive bacteria.

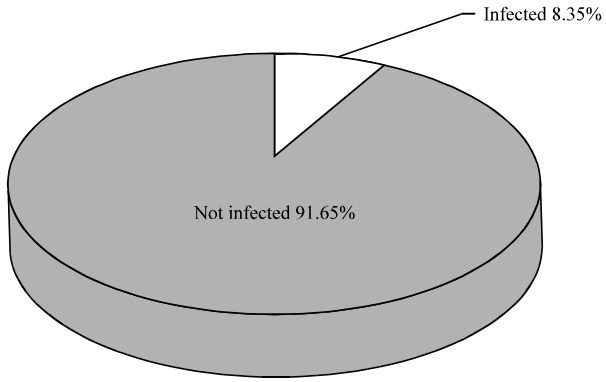

Fig. 1: Neonatal sepsis infection rate (n = 1365)

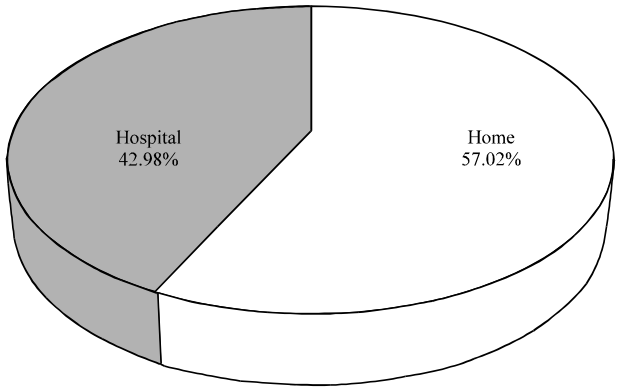

Fig. 2: Birth place of the infected child

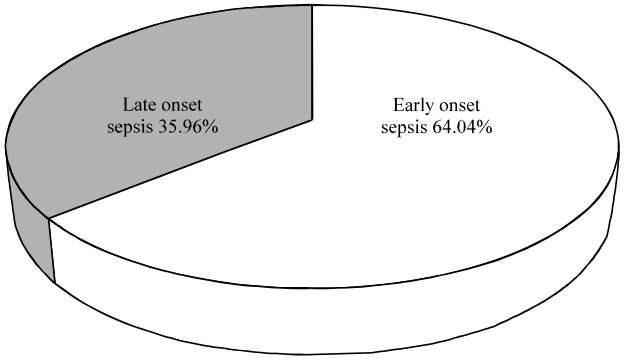

Fig. 3: Types of neonatal sepsis infection

Table 1: Isolated microorganisms

Organisms	ESBL positive cultures (n = 22)	ESBL negative cultures (n = 90)	Percentage of total isolates (n = 114)	Early onset sepsis (n = 73)	Late onset sepsis (n = 41)
Klebsiella pneumoniae	22 (27.85%)	57 (72.15%)	67.30%	50	29
Serratia marcescens	0 (0%)	19 (100%)	16.67%	11	08
Acinetobacter spp.	0 (0%)	8 (100%)	7.02%	06	02
Pseudomonas aeruginosa	0 (0%)	4 (100%)	3.51%	04	00
Proteus mirabilis	0 (0%)	2 (100%)	1.75%	01	01
Staphylococcus aureus	N/A	N/A	1.75%	01	01

Biochemical characteristics: Kligler Iron Agar (KIA), Motility, Indole and Urease (MIU), citrate and oxidase test showing typical characteristics for the five types of Gram negative bacteria, where coagulase and catalase test provides positive result for only one Gram positive bacteria.

API scores (only for *Klebsiella pneumoniae*): To use the Analytical Profile Index (API), the biochemical profiles obtained are transformed in to a numerical profiles i.e., a number which enables the easy transcription of all the results obtain for an organism and comparison with the profiles listed in the index. The principle of coding is to condense the binary pieces of information (+ or -) into a numerical profile. To do so the tests are divided into groups of three and each positive reaction is given a value equal to 1, 2 or 4 according to the position of the test in its group: First, second or third, respectively. The sum of these values (0 for negative reactions) given the corresponding digit with a value between 0 and 7. The API 20 E strip consists of 20 tests separated into 7 groups.

Antibiogram of the isolated Gram negative organisms: Ninety six percent (107) isolates were ampicillin resistant, 88% (99) were gentamicin (CN) resistant, 84% (94) were cotrimoxazole (SXT) resistant, 33% (37) were cephalexin (CFL) resistant, 37% (41) were cephradine (CED) resistant, 31% (35) were ceftazidime (CAZ) resistant, 35% (39) were amoxiclav (AMC) resistant, 9% (10) were ciprofloxacin (CIP) resistant, 8% (09) were ceftriaxone (CRO) resistant, 3% (03) were amikacin (AK) resistant, 0.0% (0) were imipenem (IPM) resistant (Fig. 4). On the other hand less than five percent (05) isolates were intermediate and 0% (0) were sensitive against ampicillin (AMP), 9% (10) and near about 3% (3) were intermediate and sensitive, respectively against gentamicin (CN), 12.5% (14) and 3.5% (4) strains were intermediate and sensitive correspondingly against cotrimoxazole (SXT), 54% (61) and 12.5% (14) were intermediate and sensitive in that order against

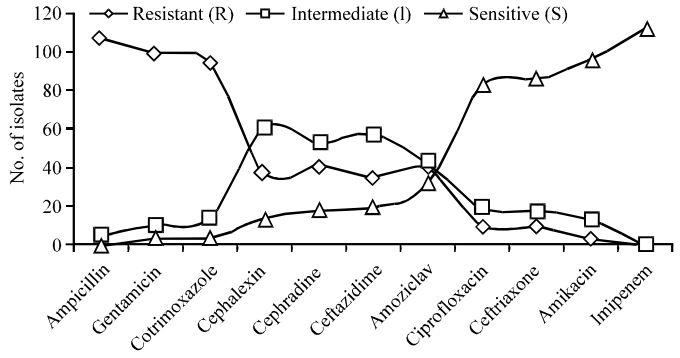

Fig. 4: Antibiogram of Gram negative organisms

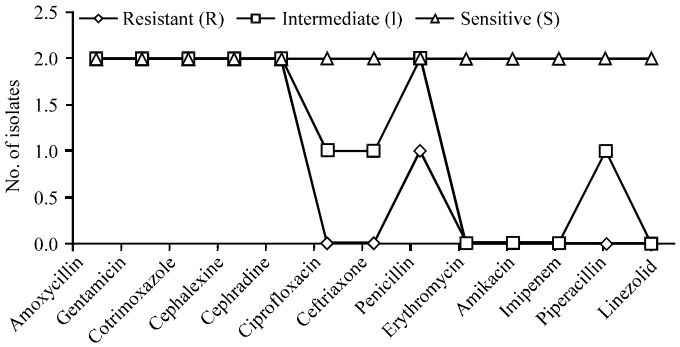

Fig. 5: Antibiogram of Gram positive organisms

cephalexin (CFL), 47% (53) and 16% (18) were intermediate and sensitive, respectively against cephradine (CED), 51% (57) and 18% (20) were intermediate and sensitive, respectively against ceftazidim (CAZ), 37.5% (42) and 28% (31) were intermediate and sensitive, respectively against amoxiclav (AMC), 17% (19) and 74% (83) were intermediate and sensitive, respectively against ciprofloxacin (CIP), 15% (17) and 77% (86) were intermediate and sensitive, respectively against ceftriaxone (CRO), 12% (13) and 86% (96) were intermediate and sensitive, respectively against amikacin (AK), 0% (0) and 100% (112) were intermediate and sensitive, respectively against imipenem (IPM).

Antibiogram of the isolated Gram positive organisms: Total two (02) cases of *Staphylococcus aureus* infection were identified during the surveillance period. All of the isolates (100%) showing resistance to amoxycillin (AMO), gentamicin (CN), cotrimoxazole (SXT), cephalexin (CFL) and cephradine (CED). On the other hand 50% (01) were resistant and 50% (01) were intermediate, respectively against Penicillin (P), where Piperacilin (PIP) were 50% (1) intermediate and 50% (1) showed sensitive agains the organism. In case of, ciprofloxacin (CIP) and ceftriaxone (CRO) 50% (01) were intermediate and 50% (01) were sensitive (Fig. 5). Erythromycin (ERY), linezolid (LZO), amikacin (AK) and imipenem (IPM) all are 100% (02) sensitive to *Staphylococcus aureus*.

MDR results: In the present study, we found that as a whole 84% (96) isolates were multidrug resistant out of 114 isolates (Fig. 4-6).

ESBL of the isolates: Interpretation of ESBL was based on extended zone of inhibition between double disk containing amoxicillin+clavulanic acid and third generation ceftazidime (CAZ)

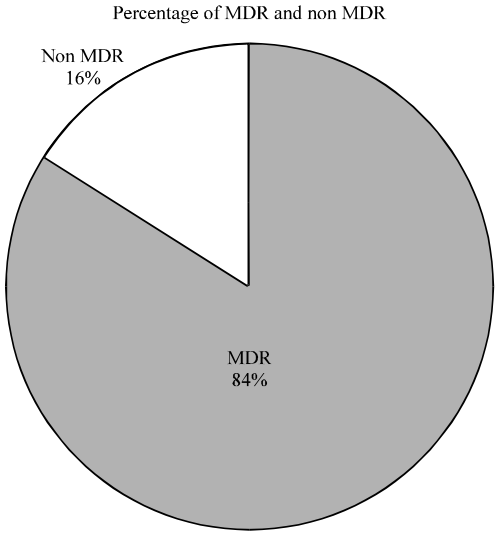

Fig. 6: Multidrug resistance pattern of the isolates

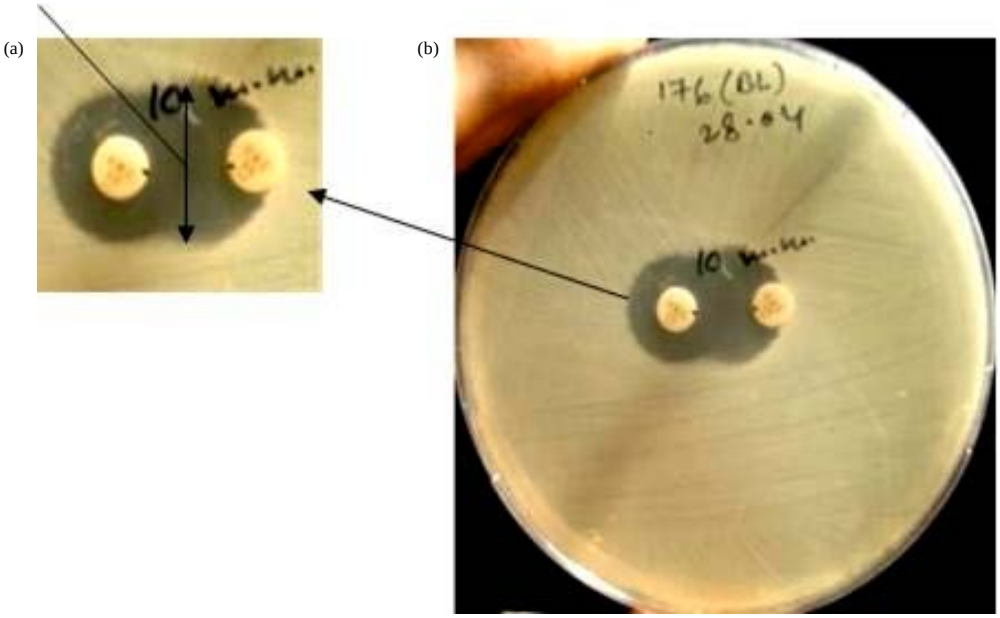

Fig. 7(a-b): ESBL result at 10 mm distance. Extended zone produce by Double Disc (DD) and third generation cephalosporin (3GC)

(Fig. 7, 8). Production of zone designated as ESBL positive. Further, the ESBL result was compared with the zone of CAZ at antibiogram. Among the all isolated organisms only *Klebsiella pneumonia* detected as ESBL producer. The 27.85% *Klebsiella pneumoniae* detected as ESBL positive while 72.15% revealed as ESBL negative out of 79 isolates. All of the CAZ resistant (35) are ESBL non producers while 53 out of 57 intermediate are ESBL negative and 02 out of 2 sensitive also showed ESBL negative properties (Table 2).

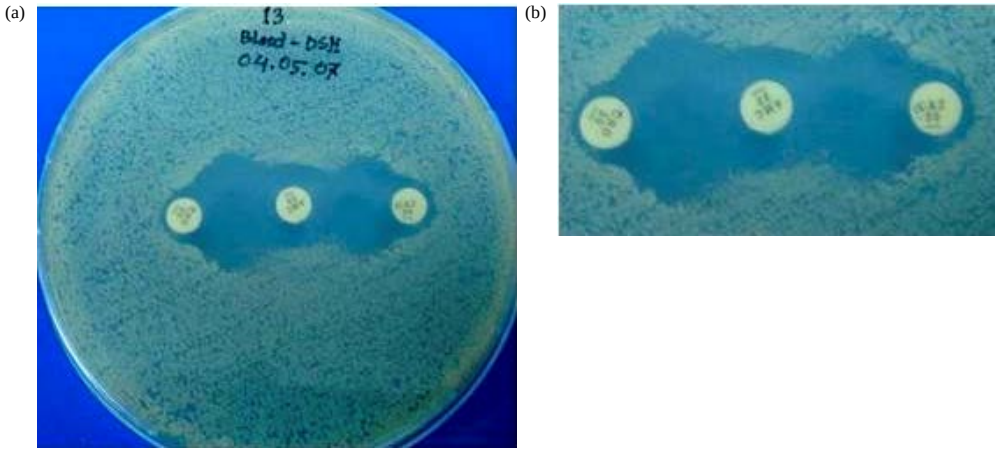

Fig. 8(a-b): ESBL results at 15 mm distance. Extended zone produce by Double Disc (DD) and third generation cephalosporin (3GC)

Table 2: Baseline characteristics of the study

Characteristics	ESBL-producing organisms (n = 22)	Non-ESBL-producing organisms (n = 92)
Onset of sepsis		
Early onset sepsis	22	51
Late onset sepsis	00	41
Sex		
Male	14	58
Female	08	34
Gestational age		
Full term babies	09	46
Preterm babies	13	46
Birth weight		
<1500 g	14	21
1500-2499 g	08	42
≥2500 g	00	29
Clinical signs of sepsis		
Symptomatic	22	58
Asymptomatic	00	25
Various focal findings	00	09
Maternal features/mothers receiving intrapartum antibiotic: 35 (30.71%) had received intrapartum antibiotics		
Premature rupture of membrane	10 (28.57%)	
Prematurely	20 (57.14%)	
Prolonged rupture of membrane	02 (5.71%)	
Fever	03 (8.57%)	
Mortality		
Survival	20	92
Death	02	0
Mode of delivery		
Vaginal delivery	12	50
Cesarean	10	37
Assisted delivery	00	05

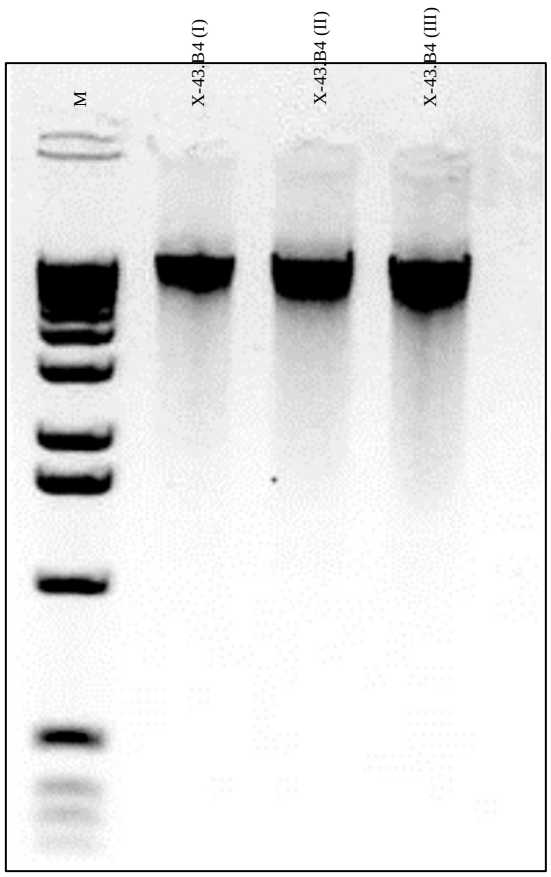

Fig. 9: Three plasmids, the size of plasmid could be more than 20 kbp

Plasmid of ESBL producing *Klebsiella pneumoniae*: This study only detected the occurrence of plasmid in *Klebsiella* but could not separated different types of plasmid in the cell. In the Fig. 9, the bands were failed to separate intensely on the gel due to very similar size of plasmids between them. Further experiment will be continued to isolate separate and intense band of plasmids.

PFGE of ESBL producing *Klebsiella pneumoniae*: All randomly selected cases that assumed as ESBL producing neonatal sepsis infection were analyzed by pulsed field gel electrophoresis. Lambda DNA standard were used as a marker in the first lane (Fig. 9) and three isolated strains were analyzed to perceive the clonal relation between the strains which were isolated from the neonates during the surveillance period. All of the three strains gave the same patterns of band on PFGE. Figure 9 represents the banding pattern where each of the three strains was revealed 17 bands in a same position. That means, all three strains (Fig. 10) have similar type of DNA sequence at restriction site which exposed same types of fragment after treatment with restriction enzyme. It may indicate that, all of the three neonatal sepsis infection may be originated from the same clone.

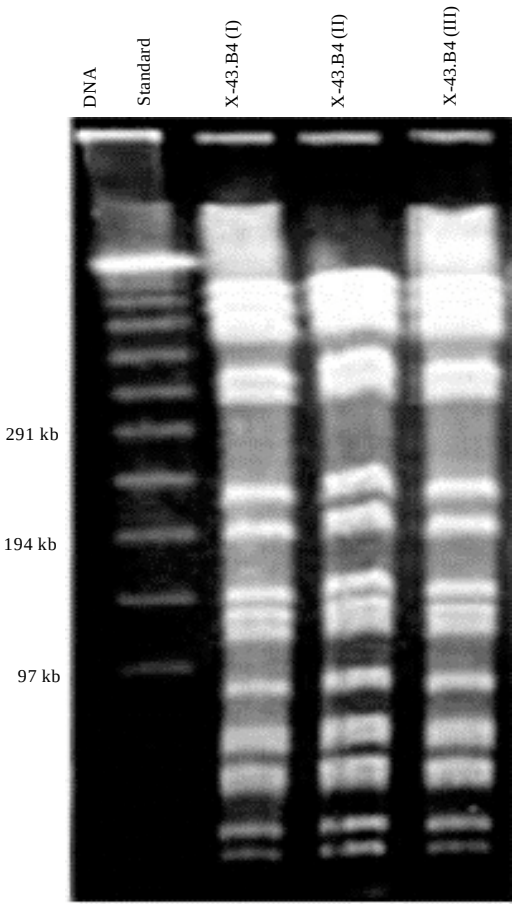

Fig. 10: Left most well inserted with standard DNA plug mold, the 2nd well from the left inserted with X-43.B4 (I), 3rd well with X-43.B4 (II), 4th well inserted X-43.B4 (III) isolated from the blood culture during the surveillance period

DISCUSSION

Our surveillance clearly confirmed that neonatal sepsis infections are an important clinical problem in the Chittagong area of Bangladesh. We have found that (Fig. 1), 8.35% of the all neonates (n = 1365) examined for sepsis infection were infected, a previous study has reported that 7.4% sepsis infection (Khadilkar *et al.*, 1995). In this study, we reported that early onset sepsis was more common 64.04% then late onset sepsis 35.96% which was compatible with the reports from the other developing countries (Vesikari *et al.*, 1989). Among the culture positive cases, 42.98% were born in hospital and 57.02% were not born in hospital. We also found male predominance, male accounted for 63.16% and female 36.84% with the male to female neonates ratio 1.71:1 the results are parallel with previous findings (Ahmed *et al.*, 1998). In the present study, prematurity and low birth weight was the leading cause of sepsis infection the findings are consistent with the previous results from other parts of Bangladesh (Bhatia, 1989). In this study, we reviewed the maternal charts of the infected child among them 30.70% had received intrapartum antibiotics which are close together the reports of Bangladesh maternal health services and maternal mortality

survey in 2001. The use of ampicillin as a prophylactic agent used in the present study increased ampicillin resistant neonatal sepsis infection while GBS declined, similar reports have been found from England hospital (Schrag *et al*., 2000). The previous investigation on neonatal sepsis suggest that *Klebsiella* as the predominant pathogen (Ahmed *et al*., 2002) in Bangladesh. Our results showed an apparent dominance of *Klebsiella pneumonia* providing further evidence. In this study, the mortality rate was 1.75% which demonstrated the major impact of preterm delivery and low birth weight. Various mortality rates from 2-14% have been reported from other Asian countries (Vesikari *et al*., 1989; Stoll, 1997; Waheed *et al*., 2003). Mortality rate in early onset sepsis was more common then late onset sepsis. ESBL producing *Klebsiella pneumonia* was the major pathogen with highest mortality rate in this study (02 out of 02 patients died). The results are consistent with the data from other studies (Greenwood *et al*., 1987). In the present study, both Gram positive and Gram negative showed sensitivity against the antibiotic imipenem (100% sensitive). Similar observations have been made by previous group of workers (Ahmed *et al*., 1998). In this study, maximum sensitivity (100%) was observed in imipenem and linezolid; these two drugs should not be used indiscriminately and be kept as a reserve drugs, otherwise resistance to these drugs may develop, thereby threatening the treatment. In this study, we found that among the all isolated Gram negative organisms only *Klebsiella pneumonia* produces ESBL. To our knowledge no previous studies have been conducted in the Chittagong area of Bangladesh to detect ESBL. Resulting, our study showed an apparent existence of ESBL producing *Klebsiella pneumonia* within the region. However, the results are consistent with the other parts of Bangladesh (Rahman *et al*., 2004) and in geographical context (Chandel *et al*., 2011; Kim *et al*., 2002). Furthermore, plasmid analysis and molecular characterization of three ESBL producers was done to perceive the clonal relation between the strains of *Klebsiella pneumonia*. It has been shown that all the three strains gave the same patterns of band on Pulsed Field Gel Electrophoresis (PFGE). Results indicated that all the ESBL producing neonatal sepsis infection may be originated from the same clone. This is in harmony with other studies (Kim *et al*., 2002).

In conclusion, this study provides evidence that neonatal sepsis infection represent a serious clinical problem in the Chittagong area of Bangladesh. This investigation showed epidemiological characteristics associated with infection. Most of the organisms showed resistance to commonly used antibiotics and ESBL producing bacteria also have been reported. PFGE showed the clonal relation between the strains. Keeping in mind the high prevalence of ESBL producing bacteria in this study, we feel it is very important to implement a revised strategy of empirical therapy and more monitoring should be conducted in the future to react rapidly in case of changes in susceptibility pattern and occurrence of outbreaks.

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