

Intestinal carriage of multidrug-resistant gram-negative bacteria in preterm-infants during hospitalization in neonatal intensive care unit (NICU)

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The prevalence and antibiotic susceptibility of intestinal carriage of Gram-negative bacteria among preterm infants admitted to the neonatal intensive care unit (NICU) in a tertiary teaching hospital in Malaysia were determined. A total of 34 stool specimens were obtained from preterm infants upon admission and once weekly up to two weeks during hospitalization. The presumptive colonies of *Escherichia coli* and *Klebsiella pneumoniae* were selected for identification, antibiotic susceptibility testing, and subtyping by using pulsed-field gel electrophoresis (PFGE). Out of 76 Gram-negative isolates, highest resistance was detected for amoxicillin/clavulanate (30.8%, $n = 16$), ceftriaxone (42.3%, $n = 22$), ceftazidime (28.8%, $n = 15$), cefoxitin (28.8%, $n = 15$), aztreonam (36.5%, $n = 19$), and polymyxin B (23.1%, $n = 12$). Three colistin resistant *K. pneumoniae* have also been detected based on *E*-test analysis. Thirty-nine isolates of *K. pneumoniae* and 20 isolates of *E. coli* were resistant to more than three antimicrobial classes and were categorized as multidrug resistant (MDR). PFGE analysis revealed a higher diversity in pulsotypes for *K. pneumoniae* (18 pulsotypes) in comparison to *E. coli* (four pulsotypes). In addition, a total of fifteen pulsotypes was observed from 39 MDR *K. pneumoniae*. The risk factors for antibiotic resistance were assessed using random forest analysis. Gender was found to be the most important predictor for colistin resistant while length, OFC, and delivery mode were showing greater predictive power in the polymyxin B resistance. This study revealed worrying prevalence rates of intestinal carriage of multidrug-resistant *K. pneumoniae* and *E. coli* of hospitalized preterm infants in Malaysia, particularly high resistance to polymyxins.

Keywords: Preterm infants, Meconium, Multidrug resistant, Gram-negative enteric, PFGE

Introduction

Infants admitted to neonatal intensive care units (NICUs) are at high risk for developing healthcare-associated infection due to the severity of their illness and exposure to invasive medical devices. Healthcare-associated neonatal infection caused by multidrug-resistant Gram-negative (MDRGN) bacilli has emerged as a substantial health problem in developing countries.^{1,2} Several studies have been carried out to characterize the epidemiology of nosocomial MDRGN by identifying risk factors for their acquisition and to trace horizontal transmission between newborns.³⁻⁶ However, the studies have yielded quite different findings because the transmission of MDRGN is

mainly dependent on the interaction between human and environmental ecology of NICUs.⁵

The infections with Gram-negative bacilli that are resistant to multiple antibacterial drugs have been increasingly reported in NICUs.^{2,7} This is mainly due to empiric treatment with antimicrobials in the NICUs. Repeated courses of antibiotic treatment and long-term exposure to antibiotics have resulted in an increase in antibiotic-resistant organisms.^{8,9} In addition, antibiotics are also administered to preterm infants who are at risk of infections soon after birth, especially those with very low birth weight.¹⁰

The frequent use of broad-spectrum antibiotics in hospital has given rise to the selection of resistant strains.¹¹ It has also been recognized that antibiotic resistance in the nosocomial setting is an important factor that influences

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the neonatal outcomes. For instance, high fecal carriage rates have been identified as a significant risk factor associated with ESBL *K. pneumoniae* infections.¹² Nevertheless, few studies have elucidated such risk in neonatal ESBL *Klebsiella* spp. colonization.¹³ In Malaysia, MDRGN infection among infants remains a major threat. Previously, Ariffin *et al.*⁷ have defined the pattern of antibiotic resistance amongst Gram-negative bacilli in NICU and PICU, where all isolates showed low susceptibility rates to the third generation cephalosporins (47–62%) and more than 50% of *K. pneumoniae* and *E. coli* isolates were extended-spectrum beta-lactamase (ESBL) producing strains.⁷ *K. pneumoniae* is among the commonest Gram-negative bacteria causing late-onset bacteremia in the NICU of a tertiary teaching hospital located in northeastern Malaysia.¹⁴ This pathogen was associated with nosocomial infection and high infection rates among ventilated infants.^{15–17}

The present study was conducted in a tertiary level III NICU in Malaysia. The objectives were to determine the antimicrobial susceptibility patterns and pulsotypes of *K. pneumoniae* and *E. coli* isolated from the NICU where broad-spectrum antibiotics are frequently used. The outcome of the antibiotics currently used for empirical therapy was also recorded and associated with the demographic and clinical characteristics of the infants.

Materials and methods

Patient and study design

Setting

Preterm infants (gestational age of less than 37 weeks) admitted to the NICU of a tertiary hospital in Kuala Lumpur, Malaysia, were prospectively enrolled and sampled. No exclusion criterion was applied to the preterm infants group. This study did not involve any intervention apart from the routine medical procedures, but the major demographic and clinical data for each participant were extracted from the hospital records (Table 1). Informed consents were obtained from parents and the study protocol was approved by the institutional ethics committee (MEC ID: 201310-0267). Confidentiality and anonymity of the subjects were maintained, and sample collection was carried out according to institutional ethics guidelines.

Collection of specimens

Stool specimens were obtained within 48 h of admission, and on the 7th day and 14th day of life during their stay in the NICU. All the samples were stored at –30 °C until analysis.

Microbiological methods

Isolation of Gram-negative bacteria

Stool specimens were diluted 1:10 in phosphate-buffered saline (PBS) and inoculated onto CHROMagar Orientation plates (CHROMagar™ Orientation; Becton Dickinson, Heidelberg, Germany). The plates were incubated aerobically at 37 °C. After 20 h, the plates were examined

for growth and colony characteristics. *E. coli* presented pink colonies while *K. pneumoniae* exhibited metallic blue colonies on the CHROMagar Orientation plates. Three to five colonies for each distinct color group were randomly selected and cultured on a non-selective agar.

Identification

Overnight culture of the isolates was suspended and washed in PBS. Cells were centrifuged at 13,000 rpm for two minutes to pellet the cells. A volume of 200 µL of ultrapure water was added to each tube. The cells were then subjected to heating at 99 °C for 10 min and snapped cool on ice for 5 min. The PCR specific identification of *E. coli* was performed using the primers *phoA1* (5'GTG ACA AAA GCC CGG ACA CCA TAA AT 3') and *phoA2* (5'TAC ACT GTC ATT ACG TTG CGG ATT TGG 3')¹⁸ while identification of *K. pneumoniae* was performed using the primer pair *mdh1* (5'CCC AAC TCG CTT CAG GTT CAG 3') and *mdh2* (5'CCG TTT TTC CCC AGC AGC AG 3').¹⁹ PCRs for the identification of *E. coli* and *K. pneumoniae* were performed in Veriti® 96-Well Fast Thermal Cycler (Applied Biosystems, California, USA) as previously described by Kong *et al.*¹⁸ and Diancourt *et al.*¹⁹, respectively. Gel electrophoresis was carried out by applying 2 µL of PCR products to 1% agarose gel. Gels were run for 30 min at 100 V in 0.5x TBE (tris-borate-EDTA) buffer. DNA molecular weight marker 100 bp DNA ladder (Promega, Wisconsin, USA) was used as size standard. Gels were visualized by UV illumination after staining with SYBR® Safe DNA Gel Stain (Life Technologies, CA, USA).

Determination of susceptibility

Susceptibility testing was performed by disk diffusion test according to the CLSI guidelines.²⁰ The 0.5 McFarland (1.0 for mucoid phenotype) of test organism was uniformly inoculated over the Mueller-Hinton agar (MHA). Susceptibilities of the isolates toward 14 antibiotics such as amoxicillin (10 µg), amoxicillin/ clavulanic acid (20/10 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefoxitin (30 µg), aztreonam (30 µg), imipenem (10 µg), meropenem (10 µg), gentamicin (10 µg), tobramycin (10 µg), ciprofloxacin (5 µg), polymyxin B (300unit), colistin sulfate (polymyxin E; 10 µg), and chloramphenicol (30 µg) were determined. *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *K. pneumoniae* ATCC 700603 were used as quality control strains for antibiotic susceptibility testing. Isolates showing intermediate antimicrobial susceptibility were considered to be resistant. Isolates were assigned as multiple drug resistance (MDR) when they are resistant to at least one member from three different antibiotic classes.²¹ In addition, based on the CLSI guidelines, putative ESBL producers were determined.

E-test for colistin-resistant strains

The colistin E-test (AB Biodisk; Biomérieux, Sweden) was performed on MHA according to the manufacturer's

Table 1 Demographic and clinical information of the infant cohort

Infant	Gestational age (weeks)	Race	Gender	Birth weight (g)	Birth length (cm)	Birth head of circumference (cm)	Maternal GPA	Delivery mode	Sample collection (day of life)	Isolation of <i>K. pneumoniae</i>	Isolation of <i>E. coli</i>
B1	33	Chinese	Female	1570	40	28	G1P0A0	Cesarean section	0, 7, 14	Positive	Positive
B2	33	Chinese	Male	1700	39	28	G4P2A1	Cesarean section	0, 7	Positive	Positive
B4	32	Malay	Male	1505	38	28	G1P0A0	Vaginal	0, 7, 14	Negative	Negative
B6	30	Chinese	Female	1390	37	28	G1P0A0	Cesarean section	0, 7, 14	Positive	Negative
B7	30	Chinese	Female	490	28	21	G1P0A0	Cesarean section	0, 7, 14	Negative	Negative
B8	30	Malay	Male	1295	37	26.5	G1P0A0	Vaginal	0, 7, 14	Positive	Positive
B9	35	Indian	Female	1455	40	28	G2P1A0	Cesarean section	0, 7, 14	Positive	Positive
B11	36	Malay	Female	1555	37	29.2	G1P0A0	Cesarean section	0, 14	Positive	Positive
B12	34	Chinese	Female	2845	45	33	G3P2A0	Cesarean section	0	Negative	Negative
B13	32	Malay	Female	1420	41	26.5	G3P0A2	Cesarean section	0, 7, 14	Positive	Positive
B14	32	Malay	Female	785	32.5	25.5	G3P0A2	Cesarean section	0, 7, 14	Positive	Positive
B15	32	Malay	Female	1540	38	28	G1P0A0	Cesarean section	0	Negative	Negative
B16	30	Malay	Female	902	34	25	G1P0A0	Cesarean section	0	Negative	Negative
B17	30	Malay	Female	1240	39	26.5	G1P0A0	Cesarean section	0	Positive	Negative
B18	30	Malay	Male	1220	37.5	26.3	G1P0A0	Cesarean section	0	Positive	Negative
B19	33	Indian	Male	1905	41	28	G1P0A0	Vaginal	0	Negative	Negative

procedures guidelines. The MIC was determined at the intersection of the inhibition zone with the strip. Resistant strains were defined as isolates with MICs for colistin > 2 mg/L using the CLSI breakpoint for *Pseudomonas aeruginosa*.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed according to established protocol from PulseNet USA.²² Genomic DNA of *E. coli* and *K. pneumoniae* was digested with *XbaI* restriction enzyme (12 U per plug) (Promega, Wincousin, USA). *XbaI*-digested *Salmonella* Braenderup (H9812) was used as the DNA size marker. Pulsotypes were analyzed using BioNumerics v6.0 software (Applied Maths NV, Sint-Martens-Latem, Belgium). The variability of the strains was determined on the basis of the Dice coefficient of similarity (*F*) and the unweighted pair group method with arithmetic averages (UPGMA) clustering analysis at 1.5% position tolerance. Interpretive clustering of the PFGE patterns was based on the banding patterns and the variability of the isolates as previously described by Barrett et al.²³

Risk assessment

Eleven demographic variables, including gestational age, gender, race, birth weight, length, head circumferences, delivery mode, maternal gravida/para/abortus (GPA), and breastfeeding were tested as independent variables in the classification of the antibiotic resistance profiles such as resistant to at least three beta-lactam antibiotics, resistant to polymyxin B, resistant to colistin (based on *E-test*), and resistant to gentamicin. Random forest was carried out using Rattle R program package to identify and validate predictors with respect to the risk factors for antibiotic resistance.²⁴ This recursive partitioning method is selected as it has been shown to perform well under the small sample size and relatively larger categorical predictors problems.²⁵

Results

Microbiological methods

In total, 34 stool specimens from 16 infants were collected. Most of the meconium samples were unculturable using selective agar. *K. pneumoniae* and *E. coli* were isolated from ten (29.4%) and seven (20.6%) specimens, respectively. Demographic and clinical information of the infant cohort were also extracted and tabulated in Table 1. In total, 24 *E. coli* and 52 *K. pneumoniae* were isolated, identified and tested for antimicrobial resistance. Disk diffusion test was carried out on the *K. pneumoniae* and *E. coli* isolates (Table 2). For *K. pneumoniae* isolates, high resistance rates were detected for amoxicillin/ clavulanate (30.8%, *n* = 16), ceftriaxone (42.3%, *n* = 22), ceftazidime (28.8%, *n* = 15), cefoxitin (28.8%, *n* = 15), aztreonam (36.5%, *n* = 19), and polymyxin B (23.1%, *n* = 12). Compared to *K. pneumoniae*, *E. coli* isolates were overall more resistant

Table 2 Antimicrobial activity of 16 antibiotics tested against *K. pneumoniae* and *E. coli* by disk diffusion test

Antibiotics	No. of <i>K. pneumoniae</i> isolates (n = 52)			No. of <i>E. coli</i> isolates (n = 24)		
	Susceptible	Resistant	Resistance rate (%)	Susceptible	Resistant	Resistance rate (%)
Amoxicillin (AML)	0	52	100	2	22	91.7
Amoxicillin/clavulanic acid (AMC)	36	16	30.8	23	1	4.2
Ceftriaxone (CRO)	30	22	42.3	4	20	83.3
Ceftazidime (CAZ)	37	15	28.8	7	17	70.8
Cefoxitin (FOX)	37	15	28.8	23	1	4.2
Aztreonam (ATM)	33	19	36.5	4	20	83.3
Imipenem (IMP)	47	5	9.6	22	2	8.3
Meropenem (MEM)	49	3	5.8	23	1	4.2
Gentamicin (CN)	43	9	17.3	24	0	0
Tobramycin (TOB)	45	7	13.5	24	0	0
Ciprofloxacin (CIP)	48	4	7.7	24	0	0
Polymyxin B (PB)	40	12	23.1	23	1	4.2
Colistin sulfate (CT)	45	7	13.5	24	0	0
Chloramphenicol (C)	0	52	100	24	0	0

to the tested third generation cephalosporins such as ceftriaxone (83.3%, $n = 20$) and ceftazidime (70.8%, $n = 17$). High resistance rates were also observed for amoxicillin (91.7%, $n = 22$) and aztreonam (83.3%, $n = 20$). None of the isolates was resistant to gentamicin, tobramycin, ciprofloxacin, colistin, and chloramphenicol. Eight *K. pneumoniae* and three *E. coli* isolates were resistant to either imipenem or meropenem. Seven *K. pneumoniae* isolates showed resistance to colistin based on the disk diffusion method and were further confirmed by using *E*-test. Out of the seven colistin-resistant isolates tested using *E*-test, three isolates were confirmed as colistin-resistant with the MIC more than 256 $\mu\text{g}/\text{mL}$. Based on the CLSI initial screening test for Extended Spectrum Beta-Lactamase (ESBLs), 24 isolates of *K. pneumoniae* and 21 isolates of *E. coli* were identified as putative ESBL-producers, subject to further confirmatory tests. Twenty isolates of *K. pneumoniae* and six isolates of *E. coli* were resistant to more than three antimicrobial classes, hence categorized as MDR strains.

Pulsed-field gel electrophoresis

For isolates from the same specimen, only those showing distinct antibiogram were subjected to PFGE. Isolates from the same specimen with identical antibiotic profile were omitted in this experiment. Among the 37 selected isolates, *K. pneumoniae* yielded only 18 different pulsotypes (KPXbaI 1–18; Fig. 1) and one isolate from B2 was untypable. Isolates from specimens of infant B9 (K11, K14, K16, K44, K46, K47, and K48) yielded the highest number of different pulsotypes (three pulsotypes) while isolates from infant B6 (K37, K39, and K41), B17 (K30, K57, and K58), and B18 (K33 and K35) gave variable MDR profiles despite displaying identical pulsotype. At the similarity of 80%, three major clusters were observed. Cluster A was comprised of isolates from infant B6 (day 7 and day 14) and B9 (day 14). Cluster B was the largest cluster, which mainly composed of isolates from B8 (day 7 and day 14), B13 (day 7 and day 14), and B14 (day 7

and day 14). Finally, cluster C was comprised of isolates from B8 (day 7 and day 14) and B9 (day 7 and day 14). Interestingly, the isolates from B13 and B14 although displayed different antibiotic susceptibility profiles, were sharing similar pulsotypes (KPXbaI 8, 10, and 15). The 37 MDR isolates were distributed among 15 distinct pulsotypes, indicating low levels of clonality among the MDR strains. *E. coli* isolates yielded only five different pulsotypes (ECXbaI 1–4; Fig. 2). Isolates from day 7 and day 14 collection of infant B1 showed same pulsotype but different antibiograms. For instance, isolate E1 was multidrug resistant (MDR) while E3 was only resistant to imipenem. ECXbaI 1 was the most common pulsotype which shared by isolates from B8 (day 14), B9 (day 14), B11 (day 14), B13 (day 7 and day 14), B14 (day 7 and day 14). Similar to *K. pneumoniae*, isolates from B13 and B14 shown high similarity and were up of the same pulsotype regardless of collection date and antibiotic susceptibility profiles.

Risk assessment

The risk factors for antibiotic resistance were assessed using random forest analysis. Using 11 demographic and clinical parameters, the predictive power of 62.64 and 81.82% was achieved in predicting the pattern of colistin and polymyxin resistance bacteria, respectively. Gender was found to be the most important predictor for the former while length, OFC, and delivery mode were showing greater predictive power in the latter (Fig. 3(A) and (B)).

Discussion

Several multi-center studies have been carried out in Malaysia which included data from pediatrics and neonatal ICUs. Ariffin *et al.*⁷ reported on resistance patterns from three intensive care units of two hospitals in 2004. The isolates showed low susceptibility rates to the third-generation cephalosporins (47–62%) compared to imipenem (90%) and ciprofloxacin (99%).⁷ In our present study, reduced susceptibility of amoxicillin/clavulanic acid

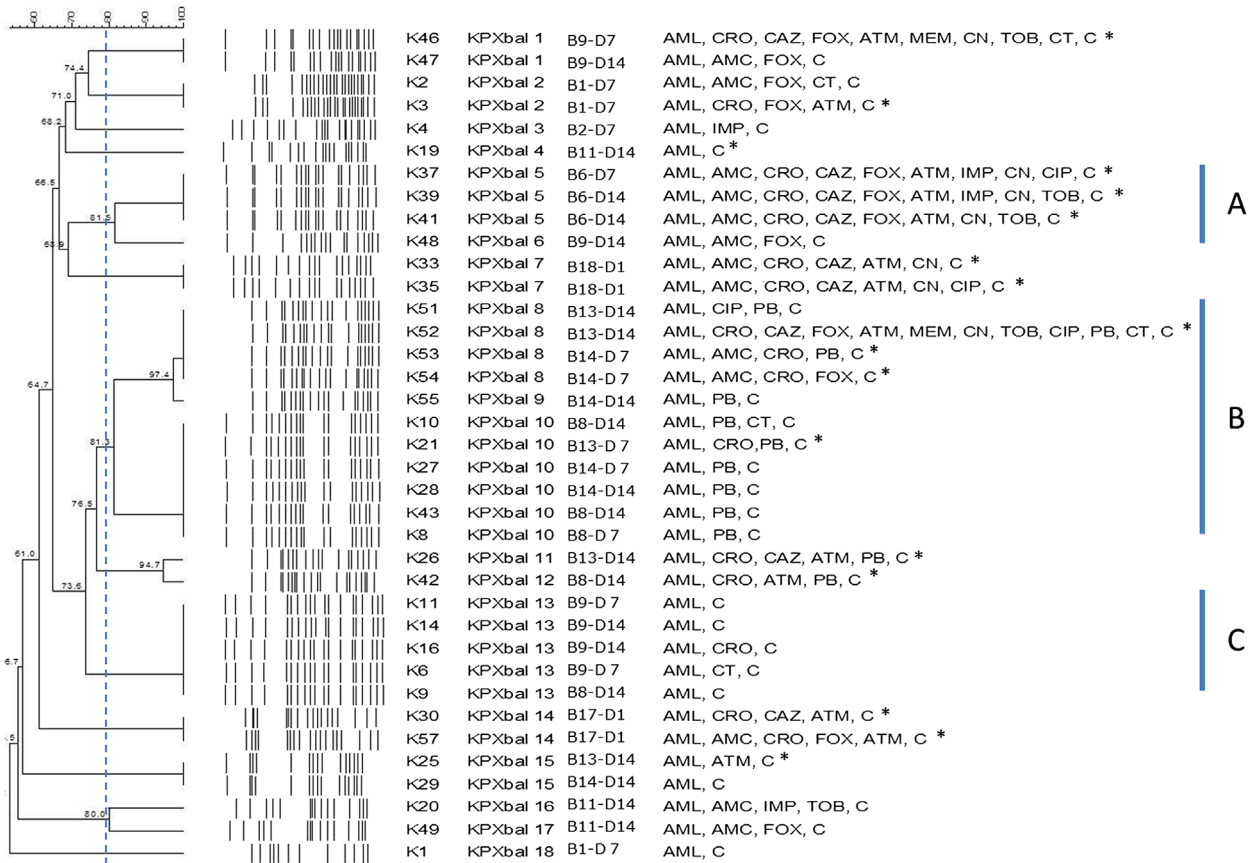


Figure 1 PFGE profiles of the non-repeated *Klebsiella pneumoniae* strains of different antibiotic susceptibility profiles that isolated from preterm infants during hospitalization.

Notes: Stool samples were collected in 3 timepoints, namely D1: day 1 (meconium); D7: day 7, and D14: day 14. Three major clusters were observed based on the similarity of 80%. Putative ESBL producers were indicated with*. CLSI recommended breakpoints for screening of *E.coli* and *K. pneumoniae* by disk diffusion for ESBL producers confirmatory test are lower than the CLSI published disk diffusion breakpoints for resistance. Thus, many isolates that have been categorized as susceptible may be considered as putative ESBL producers.

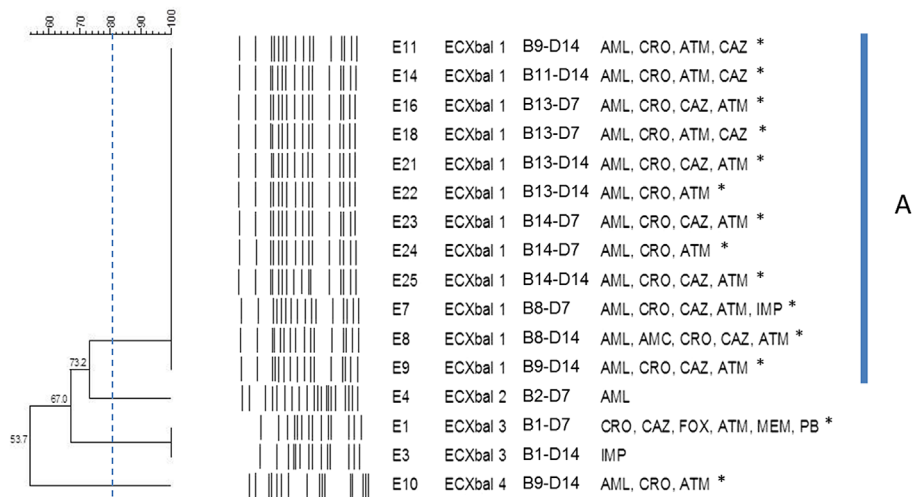


Figure 2 PFGE profiles of the non-repeated *Escherichia coli* strains of different antibiotic susceptibility profiles that isolated from preterm infants during hospitalization.

Notes: Stool samples were collected in 3 timepoints, namely D1: day 1 (meconium); D7: day 7, and D14: day 14. However, *E. coli* was not isolated from the meconium samples. Majority of the strains were of pulsotype ECXbal 1. Putative ESBL producers were indicated with*. CLSI recommended breakpoints for screening of *E.coli* and *K. pneumoniae* by disk diffusion for ESBL producers confirmatory test are lower than the CLSI published disk diffusion breakpoints for resistance. Thus, many isolates that have been categorized as susceptible may be considered as putative ESBL producers.

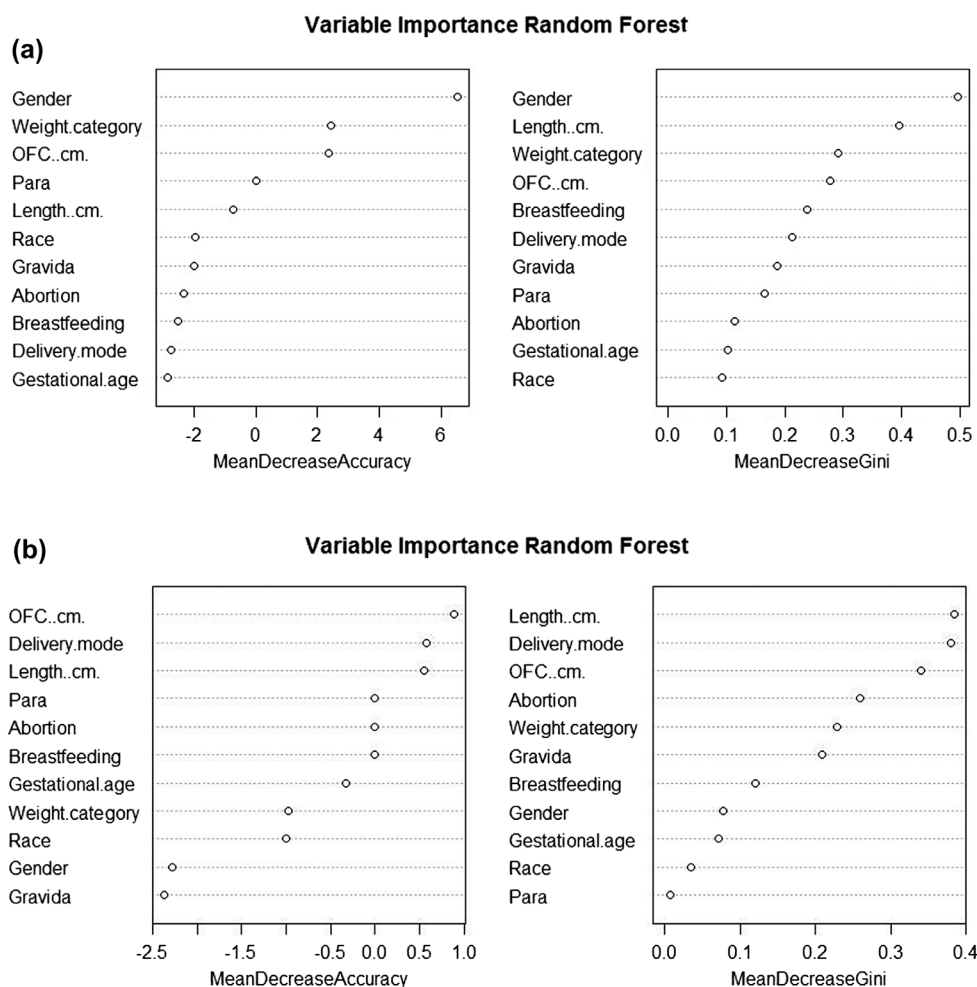


Figure 3 Variable importance based on random forest model. (A) Colistin resistance pattern, (B) Polymyxin B.

(18%) was also observed. ESBL production is a major mechanism of resistance to beta-lactam antibiotics. *E. coli* and *K. pneumoniae* are the most common ESBL producers and were identified as the causative agents of nosocomial infections. Third-generation cephalosporins resistant and ESBL selective media such as MacConkey agar plate containing 1 mg/L cefotaxime (CTX) could be used to detect ESBL/AmpC producing *Enterobacteriaceae*; however, this method was unable to detect Oxa 48-like carbapenemase producers and non-lactose fermenting *E. coli* could also be grown on the agar.²⁶ The efficiencies of ESBL detection of five ESBL selective media were determined previously and the results showed that none of them were fully ESBL selective.²⁷ Hence, in this study, CHROMagar was used as the primary screening medium as it provides good discrimination of common bacteria species in the mixed cultures isolated from clinical specimens.²⁸ Then, disk diffusion test was employed after the primary screening. In addition, ESBL-producing colonies and non-ESBL-producing colonies could be found in the same culture passage as ESBL production is usually plasmid mediated. Therefore, it was suggested that several colonies must be picked and tested from the primary culture for optimal detection.²⁹

All the results of antibiotic susceptibility tests in this study were reproducible. Based on the carbapenem susceptibility patterns, the isolates could be divided into two phenotypes namely the imipenem-resistant meropenem-susceptible phenotype and imipenem-susceptible meropenem-resistant phenotype. Weak hydrolysis of meropenem compared to imipenem has been previously reported mainly among the strains that harbored Oxa 54 and Oxa 48.^{30–32} On the other hand, the specific activity of Oxa 244 for imipenem, absence of *ompK 35*, and the defects in *ompK36* was reported to play significant roles in the occurrence of imipenem-susceptible and meropenem-resistant phenotype.^{33,34} In this study, strain E1 with cephalosporins resistance and penicillin susceptible has been observed. Amoxicillin/clavulanate is one of the widely used antibiotics, which has good activity against non-beta-lactamase-producing strains as well as some beta-lactamase producing strains.³⁵ Amoxicillin/clavulanate susceptible ESBL strains are rare, however, has been reported previously by Kumar *et al.*³⁶ and Prakash *et al.*³⁷

Nucleic acid-based molecular typing methods such as PFGE still remain as invaluable tools in establishing clonal patterns of isolates. PFGE enables the greatest discriminatory capacity compared to other techniques, making

it a very robust tool in the study of epidemiology and public health.³⁸ In this study, no direct association was established between the resistant phenotypes and *Xba*I-pulsotypes by cluster analysis of the *K. pneumoniae* and *E. coli* isolates. The *E. coli* isolates were highly clonal yet with different patterns of resistance to antibiotics. This might be due to the phenomenon where the clonal isolates harboring the same ESBL associated genes that were differentially expressed and the loss of porins or mutations which were not located at the PFGE restricted enzymes cutting sites.^{39–41} The finding is consistent with Benacer *et al.*⁴² which showed that PFGE could not distinguish similar pulsotypes with different resistance profiles. To increase the discriminatory power of PFGE, the use of more than one enzyme was previously recommended.⁴³ Nevertheless, in this study, PFGE was able to discriminate isolates from the same individual that exhibited identical resistant profile but different pulsotypes.

Preterm infants are susceptible to Gram-negative bacilli and they are most likely to be infected during or shortly before birth from the colonized maternal genital tract.⁴⁴ Based on the PFGE results, the strains that colonised the only pair of twins, B13 and B14 were clonal (KPXbal 8, KPXbal 10, KPXbal 15). Unfortunately, intrapartum antibiotic prophylaxis of the mother could not be traced and we also failed to obtain the high vaginal swab from the mother at the time of labor, hence pathway of colonization could not be determined. The results of this study will be more meaningful, especially if we were able to assess the risk of neonatal antibiotic resistant incidence in correlation to the antibiotic pressure to mothers, especially intrapartum antibiotic prophylaxis, which has been associated with neonatal sepsis or antimicrobial-resistant.⁴⁴ In addition, similar strains (clonal) were also observed in different infants. For instance, the *E. coli* isolates of pulsotype ECXbal 1 that present in infants B8, B9, B13, and B14; *K. pneumoniae* of pulsotype KPXbal 10 in infant B8 and the twins (B13 and B14) and KPXbal 13 that observed for isolates from infants B9 and B8. These suggested the clonal spread of strains between the infants in the ward and physical contact might be most likely the mode of transmission.⁵

It has been considered that the intestinal tract and meconium were sterile at birth, and later being rapidly colonized with micro-organisms from the mother and the surrounding environment. However, several studies have shown that the meconium from healthy hosts is not sterile and gut colonization may have started before birth.^{45–47} A study on the bacterial diversity of meconium in preterm infants showed an association between low bacterial diversity in meconium and high risk to develop sepsis.⁴⁸ In the present study, the isolation rate from meconium samples was low and only *K. pneumoniae* was isolated from meconium of infants B17 and B18. This is in agreement with Moles *et al.*⁴⁹ who reported low isolation rates of *Escherichia* spp and *Klebsiella* spp in meconium of

preterm infants. To overcome the limitation of culture-dependent techniques, a more robust culture-independent approach such as next generation metagenomics analysis should be applied in future to establish the bacterial communities in these infants. This may provide new clues on the initial gut colonizers in the Asian cohort which remains less studied in comparison to the western populations.

In this study, the risks of infection/colonization by the last treatment options for Gram-negative bacteria infections have been identified. For instance, gender was found to be an important factor for colistin resistance pattern. Apart from this study, male babies were reported to be more predominant to get early-onset neonatal sepsis previously.⁵⁰ Moreover, our study also showed that delivery mode and neonate development (i.e. length and OFC) were important parameters for polymyxin B resistance pattern. A low worldwide colistin resistance at 0.9–3.3% was reported in the surveillance data recorded from 2001 to 2011.⁵¹ However, in contradiction with this previous finding, high resistance rate of colistin (6%) in this study is worrying. Furthermore, in the recent report, the identification of plasmid-mediated *mcr-1* gene in polymyxin resistant in *Enterobacteriaceae* raises the possibility of widespread dissemination of resistance.⁵²

In our present study, Gram-negative isolates detected from hospitalized preterm infants are still highly susceptible to gentamicin and meropenem despite their heavy usage in the particular unit studied. Many reports have associated the bacterial resistance with uncontrolled of antibiotic use and particularly in the treatment of neonatal sepsis.⁵³ The highest rate of resistance is often observed in settings with the highest rates of antibiotic prescription.^{54,55} *E. coli* is one of the main sources of neonatal sepsis and highly resistant to commonly used antibiotics such as amoxicillin and ceftriaxone, and relatively more sensitive to less commonly used drugs like ceftazidime.⁵⁶ However, our study provided evidence that reduced susceptibility of ceftazidime has been observed in *E. coli* isolates, which was in agreement with the findings of Yuan *et al.*⁵⁷

Polymyxin B and colistin were the two polymyxins which have been used clinically against Gram-negative bacteria. Disk diffusion was reported to have an acceptable rate of errors and was useful for screening of polymyxin resistant strain, thus disk diffusion was used to detect polymyxin B resistant strains. For colistin resistant, *E*-test was further used after the initial screening using disk diffusion. Although low sensitivity of *E*-test on detecting colistin resistant strains has been reported recently,⁵⁸ our results in this study was considered reliable as no zone of inhibition was observed for three colistin strains in two repeated experiments. However, the strains will be further subjected to the other MIC detection methods, such as broth macrodilution (TDS) and broth microdilution with polysorbate 80 (BMD-T) for further comparison and evaluation.

In addition, a bigger sample size should be included in future studies to increase the robustness of the statistical analyses.

Notwithstanding the limitation, our results affirmed the prevalence of drug resistance of *E. coli* and *K. pneumoniae* in hospitalized preterm infants in Malaysia. A continuous surveillance of antimicrobial resistance in NICU should be carried out to identify the risk for infants and to assist in the empirical antibiotic therapy decision.

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