PHYLOGENETIC AND TRANSMISSION PATTERNS OF EXTENDED-SPECTRUM β-LACTAMASE –PRODUCING ESCHERICHIA COLI AMONG NEONATAL'S GUT FLORA IN INDONESIA

Rakhmatul Binti Sulistya[1], Kuntaman Kuntaman[2], Eko Budi Koendhori[3], Terza Aflika Happy[4], Wahyu Setyarini[5]

[1] Master Program in Basic Medical Sciences, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

[2,3] Departement of Clinical Microbiology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia/Dr. Soetomo General Academic Hospital, Surabaya, Indonesia

[4] Postgraduate Program of Tropical Medicine, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

[5] Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia

ABSTRACT

This study aimed to explore the phylogenetic pattern of Extended-spectrum β-lactamase-producing Escherichia coli in the gut flora of neonatal into groups A, B1, B2, D and tracing the presence of spreading among the age group of neonatal. Multiplex PCR was conducted to classify the phylogenetic group of ESBL producing E. coli into groups of commensals (A, B1) and pathogen (B2, D). The spread of bacterial agents among the host (neonatal) was conducted by the RAPD-PCR method with two random primers. Among 34 bacterial isolates were identified among early neonatal group A (22,2%), B1 (11,1%), B2 (22,2%) D (33,3%) and postneonatal groups A (8%), B1 (4%), B2 (36%) D (40%). It was not a statistically significant difference (p=0.388). The dominance of pathogenic groups B2 and D as many as 70,6%. From 34 samples found 34 types of RAPD indicated there were not any bacterial spread among neonatal. The ESBL producing E. coli among early neonatal and postneonatal were dominated by the pathogenic group of B2 and D (total=70,6%), but they were not statistically significant. There was not any spreading of bacterial agents among individual gut flora of neonatal.

Keywords: Escherichia coli; ESBL; neonate health; infection; phylogenetic

ABSTRAK

Penelitian ini bertujuan untuk mengeksplorasi filogenetik Escherichia coli penghasil Extended-spectrum β-lactamase pada flora usus neonatus menjadi kelompok A, B1, B2, D, dan menelusuri adanya penyebaran di antara dua kelompok usia neonatus. Multiplex PCR mengklasifikasikan kelompok filogenetik E. coli penghasil ESBL menjadi kelompok komensal (A, B1) dan patogen (B2, D). Penyebaran bakteri antar inang (neonatus) dilakukan dengan metode RAPD-PCR dengan dua primer acak. Dari 34 isolat bakteri yang berasal dari neonatus dini kelompok A (22,2%), B1 (11,1%), B2 (22,2%) D (33,3%) dan kelompok pascaneonatus A (8%), B1 (4%), B2 (36%) D (40%). Tidak terdapat perbedaan bermakna (p = 0,388) antara neonatus dini dan pascaneonatus. E. coli penghasil ESBL pada neonatus dini dan pascaneonatus didominasi oleh kelompok patogen B2 dan D (total = 70,6%), tetapi secara statistik perbedaan tidak bermakna. Dari 34 sampel ditemukan 34 tipe RAPD. Artinya tidak ada penyebaran bakteri antar bayi maupun antara kelompok usia neonatus.

Kata kunci: Escherichia coli; ESBL; neonate health; infeksi; filogenetik

Correspondence: Kuntaman, Departement of Clinical Microbiology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia/Dr. Soetomo General Academic Hospital, Surabaya, East Java 60286. Email: kuntaman@fk.unair.ac.id

INTRODUCTION

The β-lactamase enzyme plays a role in deactivating the β-lactam group of antibiotic derivatives, especially the third and the fourth generation cephalosporins and monobactams (Oteo et al 2012; Rath et al 2014). The emergence of resistance to several antibiotics is found in Escherichia coli bacteria (Rath et al 2014).

The harbor of Extended-spectrum β-lactamase (ESBL) producing bacteria in the intestinal flora of infants in neonatal increases in various parts of the world. ESBL producing Enterobacteriaceae group (ESBL-E) is increasing in worldwide with the incidence of infection in the pediatric and neonatal population increasing to 9% (11% neonatal and 5% children) (Storberg 2014, Flokas et al 2017)

The infant ages up to 1 year are grouped into early neonatal (0 -7 days old), and post-neonatal (28 days-1 year) (Barfield 2016). The phylogenetic pattern of Escherichia coli is included into four main phylogenetic groups A, B1, B2, and D. This classification is based on the presence of the chuA, yja, TSPE4.C2 fragment gene (Clermont et al 2000). Groups A and B1 are commensal groups, while groups B2 and D are pathogenic groups. B2 and D groups are always dominant in various infections, such as Urinary Tract Infection (UTI) (Marialoius & Santhanam 2016), neonatal septicemia (Bok et al 2020), and significantly causes meningitis in neonatal as many as 68% (Tebruegge et al 2011). Phylogenetic groups B2 and D show more virulence genes than other phylogenetic groups (Das et al 2013).

Phylogenetic groups of pathogens B2 and D in early neonatal are acquired during the delivery process a transmission from the mother's gastrointestinal tract (Das et al 2013, Muinck et al 2011), whereas in post-neonatal baby, the spread from the surrounding environment is more favorable origin (Neu & Rushing 2011, Darmstadt et al 2011).

The colonization of pathogenic groups B2 and D in neonatal indicates the transmission from person, or environmental, especially person with infection or colonization of this pathogens (Vogel et al 2000, Manges 2016). This study explored the pattern of the phylogroups and spread among ESBL producing E. coli colonized the gut of neonatal that visited Primary Health Centre in Surabaya, Indonesia.

MATERIALS AND METHODS

This study was conducted on February 2020 - July 2020, and approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Airlangga, Surabaya No. 31/EC/KEPK/FKUA/2020. The samples were re-culture of bacterial stock in -80o C from the previous study conducted by Happy et al (2019). The sample were Escherichioa coli (E. coli) collected from gut flora of neonates (early and post neonatal age) and stocked in Laboratory of Clinical Microbiology Dr. Soetomo General Academic Hospital, Surabaya. The total 34 ESBL-producing Escherichia coli (ESBL-EC) were collected from rectal swabs of 200 babies who visited 3 Primary Health Centers in Surabaya City, namely Kali Kedinding Health Center, Sidotopo Wetan Health Center, and Dupak Health Center. The ESBL-EC isolates consisted of 9 isolates from early neonatal group and 25 post-neonatal age group (Happy et al 2019).

The bacterial confirmation test was cultured with MacConcey Agar media and incubated at 37°C for 24 hours (Kazemnia et al 2014). Isolation of bacterial DNA was carried out by boiling method at 100°C for 10 minutes, then centrifugation and supernatant were use as samples in PCR (Kazemnia et al 2014).

Triplex PCR was performed to identify the target genes, namely ChuA, YjaA, TspE4C2. PCR was run as follow: initial denaturation of 5 minutes at 94°C, 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C, and a final extension step of 7 minutes at 72°C (Clermont et al 2013).

The primers used for RAPD PCR were DAF4 (5'-CGG CAG CGC C-3 ') and M13 (5'-GAG GGT GGC GGT TCT-3') (Vogel et al 2000). The PCR for RAPD was run as follow: 94oC for 5 minutes followed by 40 cycles of 94oC for 1 minute, 50oC for 1 minute, 72oC for 1 minute (Vogel et al 2000).

RESULTS

The results from identification of phylogenetic groups of ESBL-EC among 9 isolates from early neonatal group found 2 isolates (22.2%) phylogenetic group A, 1 isolate B1 (11, 1%), B2 was 2 isolates (22.2%) and D was 3 isolates (33.3%). Among 25 ESBL-ECs from post neonatal group showed 2 isolates (8%) phylogenetic group A, 1 isolate (4%) B1, 9 isolates (36%), and D 10 isolates (40%). They were not significantly different between group (p = 0.388).

Four isolates of ESBL-EC were not identified by this method, then classified as non-group. This result showed that the domination of phylogenetic group was (B2 and D) of 24 isolates (70.6%) rather than commensal group (A and B1) with 6 isolates (17.6%). However, there was no significant difference between groups of neonates (p = 0.3).
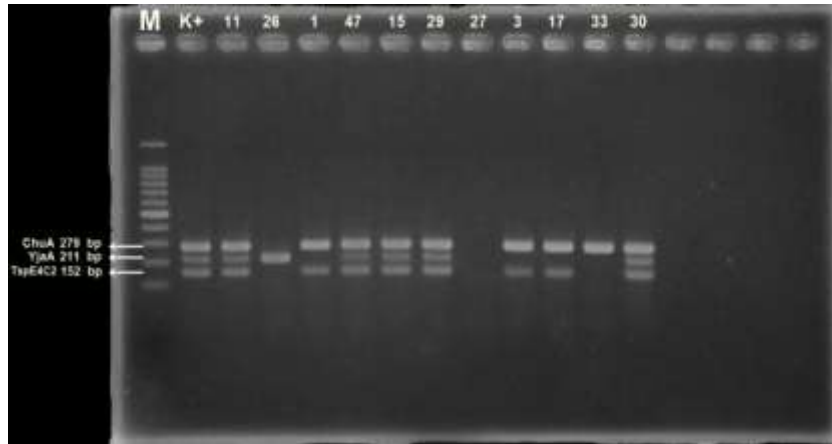

Figure 1. Results of the phylogenetic assay with ESBL-Escherichia coli Multiplex PCR in neonatal

The results of the RAPD PCR analysis showed that there were 34 types of RAPD-types from a total of 34 isolates, indicated that there was no any spread of ESBL-EC among neonates.
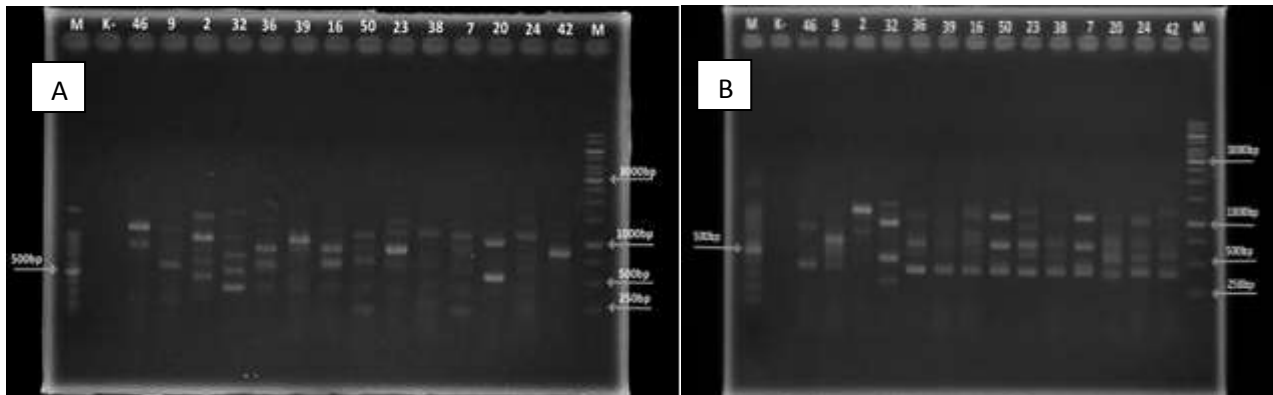

Figure 2. RAPD PCR amplification results with DAF4 primer (A) and M13 primer (B) separating DNA fragments with various sizes of ESBL producing Escherichia coli bacteria in neonatal

DISCUSSION

The study of Das et al (2013) that the antibiotic-resistant Escherichia coli group also provided a variety of phylogenetic groups which was not significant. The distribution from the 124 faecal samples was as follows: A (23 %); B1 (49 %); B2 (8 %); and D (20 %) (Das et al 2013). This study also showed various phylogenetic group without correlation between age group of neonatal.

Table 1. Phylogenetic patterns of early neonatal and post-neonatal age groups

Age group	Phylogenetic group					Total N (%)	p
	AN (%)	B1 N (%)	B2 N (%)	DN (%)	Unidentified N (%)		
Early neonatal	2 (22,2)	1 (11,1)	2 (22,2)	3 (33,3)	1 (11,1)	9 (100)	
Post-neonatal	2 (8,0)	1 (4,0)	9 (36,0)	10 (40,0)	3 (12,0)	25(100)	0,38
Total Group N (%)	4 (11,8)	2 (5,9)	11 (32,4)	13 (38,2)	4 (11,8)	34(100)	8

Early (0-7 days) and post neonatal age groups (> 28 days-1 year) are correlated with phylogenetic groups E. coli A, B1, B2, and D.

The higher prevalence of phylogenetic group B2 and D (70.6%) (Table 2) was also similar with the study of Iranpour et al (2015) and Muinck et al (2011). By the study of group B2 colonization in patients in the delivery unit showed dominant results (Muinck et al 2011), and it also concluded that pathogenic phylogroup was significantly related to the pattern of antibiotic resistance (p = 0.001) (Iranpour et al 2015). Besides, B2 phylogroups were easily colonized in the infant's intestine. It was proven that 83% of mothers with strain B2, the child also has it at an early age (2-16 days). In many countries, B2 is always dominant but

depends on geography, baby food intake, and contact time (Muinck et al 2011).

The predominance of E. coli B2 and D pathogens in early neonatal would be obtained from the mother's gastrointestinal tract during vaginal birth (Das et al 2013). A cohort study with housekeeping gene malate dehydrogenase (mdh) sequencing and 7-gene MLST between mother and baby born, prove E. coli transmission was not from nosocomial, but through the mother-child route. The strains carried by the mother were transferred to their baby born which continued to be dominant until the strains from the mother tended to decrease in the equation at the next sampling for 4 months (p = 0.0019).

Table 2. Mapping of neonatal age groups based on pathogen groups and commensal groups

Age group	Phylogenetic group			Total N (%)	P
	Non-patogen (A+B1) N (%)	Pathogen (B2+D) N (%)	Unidentified N (%)		
Early	3 (33,3)	5 (55,5)	1 (11,1)	9 (100)	
Post	3 (12)	19 (76)	3 (12)	25(100)	0,300
Total Group N (%)	6 (17,6)	24 (70,6)	4 (11,8)	34(100)	

Early (0-7 days) and post-neonatal age groups (> 28 days-1 year) are correlated with commensal groups E. coli A and B1, and pathogen groups B2 and D.

After some times, the early neonatal grow into post neonatal along with changes in the E. coli strain depending on the surrounding environmental conditions. The transfer of pathogenic microbes to neonatal through several processes of direct contact every day from delivery, breastfeeding, care from medical personnel, umbilical cord care, skincare, and direct contact with parents (Darmstadt et al 2011). The surrounding environment played a role in changing commensal strains to pathogens. Changes in virulence gene formation occurred due to competition and the transfer of genetic material among strains. Commensal strains could turn into pathogens, because they got virulence genes from surrounding strains (Muinck et al 2011).

Competition and transfer of genetic material among strains were supported by the nature of several genes in microbes that had fast-spreading characteristics in other strains; for example, one of the ESBL coding genes was CTX-M. In the study of Happy et al (2019), 30 of 34 ESBL coding genes were CTX-M. Pitout et al (2005) described the CTX-M gene in plasmids

associated with mobile elements, such as ISEcpl and sulI-type integrons, so that they could easily spread among communities. The spread in community areas was also not limited, because the spread of genes could be through animals to humans from livestock, animal products, and other reservoirs (Luo et al 2020).

The phylogenetic groups of 4 isolates could not be detected in this study. Clermont et al (2000) explained that even with the quadruplex method, a small proportion of strains in E. coli still failed to determine their phylogenetic groups. The fraction of undetermined E. coli strains represented a large-scale recombination event, where the donor and recipient belonged to two different groups. The second reason was the content of the E. coli gene which varied significantly that there might be no gene. It was not surprising that a fraction of the B1 strain could lose the TspE4.C2 DNA fragment, or that arpA could not be detected in some D strains, as did the B2 strain which failed to produce chuA products (Clermont et al 2000).

The discovery of the dominant of pathogenic phylogroups B2 and D, will increase the ability of E. coli to attack host cells, especially the role of its virulence genes. This statement was supported by the study of Das et al (2013) which referred the expression

of virulence genes through observations on the intestinal mucosa of experimental mice given several treatments. It was concluded that the virulence genes in the intestines of neonatal rats gave histopathological changes to the intestinal mucosa, and the degree of these changes was correlated with the factors of the groups whose clinical changes were maximal in groups B2 and D carrying virulence genes (Das et al 2013).

Although in this study all neonatal patients were asymptomatic, when the patient was in a low immunity condition, it would show a bad condition if it was not treated with appropriate and prompt therapy. According to Rath et al (2014), the condition of people with low awareness of hygiene could carry a risk to patients with low immunity, such as elderly patients, neonatal, and patients with multi-morbid in the distribution of antibiotic-resistant microbes. Besides, a healthy person could experience an infection for an indefinite period (Rath et al 2014).

This study found that out of 34 early and post-neonatal isolates, 34 RAPD-type (RT) groups were identified, namely RT I, II, III to XXXIV. There were no isolates with the same RT pattern through the analysis of 2 independent primers. Isolates with exactly the same RAPD amplification by chance was of little prevalence (Vogel et al 2000). This condition also explained that there was no ESBL-E. coli transmission among neonatal, either directly or indirectly in the Primary Health Center area so as an indicator of good sanitation in the health care environment, especially for neonatal patients.

Band patterns similar to the RAPD method could be applied to clinical epidemiology as long as they were correlated with the epidemiological origin of the strains. RAPD was an option that can be used to screen for the spread of E. coli in hospitals in the presence of cross-infection or epidemic suspicion (Vogel et al 2000). According to Valentine et al (2014), the spread of ESBL was caused by the spread of cellular genetic elements between a diverse population of E. coli. One of the causes of the increasing prevalence of ESBL-producing bacteria was the spread between individuals or with the surrounding environment.

The results of RAPD research in various studies had been correlated with other molecular techniques, such as PFGE, ribotyping, multiplex-PCR, and others, in which isolate amplification type equations supported epidemiological linkages through transmission process (Vogel et al 2000). The emergence of higher RAPD-type variations indicated that there was no epidemiological linkage due to the strain transmission process among neonatal patients, so that the sanitary and hygienic conditions in an area were good.

CONCLUSION

This study showed that Extended-spectrum β-lactamase producing Escherichia coli had spread to the neonatal group. Pathogenic phylogenetic groups, B2 and D, were also distributed in these groups and even showed a higher graph compared to the commensal groups, A and B1. Healthy neonatal ESBL producing E. coli pathogens could have an impact if the host had decreased immunity or mild inflammation at an undetermined time. Tracing of transmission was observed using the RAPD-PCR method which presented a higher number of RAPD –type (RT), and it showed higher genetic variations which concluded that there was no spread of strains in the health service area, so that the sanitation and hygiene in Primary Health Center had been implemented properly.

REFERENCES

Barfield WD (2016). Standard terminology for fetal, infant, and perinatal deaths. J Pediatr 137, 1-7.

Bok E, Ko˙za´nska A, Mazurek-Popczyk J, et al (2020). Extended phylogeny and extraintestinal virulence potential of commensal escherichia coli from piglets and sows. International Journal of Environmental Research and Public Health, 17,1-17.

Clermont O, Bonacorsi S, Bingen E (2000). Rapid and simple determination of the Escherichia coli phylogenetic group. Appl Environ Microbiol 66, 4555-4558.

Clermont O, Christenson JK, Denamur E, et al (2013). The clermont escherichia coli phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ Microbiol Rep 5, 58-65.

Darmstadt GL, Zaidi AKM, Stoll BJ (2011). Neonatal infection: A global perspective. Arch Dis Child Fetal Neonatal Ed 90, 221-224.

Das P, Singh AK, Mukherjee S, et al (2013). Composition of escherichia coli population in the neonatal gut: phylogroups and virulence determinants. J Med Microbiol 62, 1680-1687.

Flokas ME, Karanika S, Alevizakos M, et al (2017). Prevalence of ESBL-producing enterobacteriaceae in pediatric bloodstream infections: A systematic review and meta- analysis. PLoS One 12, 1-13.

Happy TA, Setyarini W, Ranuh IGMRG, et al (2020). Prevalence ESBL Producing Escherichia Coli among Children in Indonesia. Indian J of Public Health Research and Development, 11(05), 792-797.

Iranpour D, Hassanpour M, Ansari H, et al (2015). Phylogenetic groups of Escherichia coli strains from patients with urinary tract infection in Iran based on the new clermont phylotyping method. Biomed Res Int, 2015, 1-7.

Kazemnia A, Ahmadi M, Dilmaghani M (2014). Antibiotic resistance pattern of different Escherichia coli phylogenetic groups isolated from human urinary tract infection and avian colibacillosis. Iran Biomed J 18, 219-224.

Luo Q, Wang Y, Xiao Y (2020). Prevalence and transmission of mobilized colistin resistance (mcr) gene in bacteria common to animals and humans. Biosafety and Health 2, 71-78.

Manges AR (2016). Escherichia coli and urinary tract infections: the role of poultry-meat. Clinical Microbiology and Infection 22, 122-129.

Marialouis XA, Santhanam A. (2016). Antibiotic Resistance, RAPD- PCR typing of multiple drug resistant strains of Escherichia coli from urinary tract infection (UTI). J Clin Diagn Res, 10(3), 5-9.

Muinck EJ, De Øien T, Storrø O, et al (2011). Diversity, transmission and persistence of Escherichia coli in a cohort of mothers and their infants. Environ Microbiol Rep 3, 352-359.

Neu J, Rushing J (2011). Cesarean versus vaginal delivery: long term infant outcomes and the hygiene hypothesis. Clin Perinatol 38, 321-331.

Oteo J, Cercenado E, Fernández-romero S, et al (2012). Extended-spectrum-B- lactamase-producing Escherichia coli as a cause of pediatric infections: Report of a neonatal intensive care unit outbreak due to a CTX-M-14-producing strain. Antimicrob Agents Chemother 56, 54-8.

Rath S, Dubey D, Suhu MC, et al (2014). Surveillance of ESBL producing multidrug resistant Escherichia coli in a teaching hospital in India. Asian Pac J Trop Dis 4, 140-149.

Storberg V (2014). ESBL-producing Enterobacteriaceae in Africa a non-systematic literature review of research published 2008-2012. Infect Ecol Epidemiol 1, 1-16.

Tebruegge M, Pantazidou A, Clifford V (2011). The age-related risk of co-existing meningitis in children with urinary tract infection. PloS ONE 6, 1-6.

Valentine L, Sharp H, Hille K, et al (2014). Subgrouping of ESBL-producing Escherichia coli from animal and human sources: An approach to quantify the distribution of ESBL types between different reservoirs. International Journal of Medical Microbiology 304, 805-816.

Vogel L, Oorschot EV, Maas HM, et al (2000). Epidemiologic typing of Escherichia coli using RAPD analysis, ribotyping and serotyping. Clin Microbiol Infect 6, 82-87.