DNA SEQUENCE ANALYSIS OF HB-EGF AND CD9 GENES IN DIPHTHERIA CARRIERS AND PATIENTS IN INDONESIA

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Abstract. Diphtheria infection is a serious health problem in Indonesia. There is little data regarding the role of human genetic variability on the diphtheria infection. There is also limited information in the literature regarding the comparison between diphtheria patients and carrier state. We aimed to compare the deoxyribonucleic acid (DNA) sequences of heparin binding-epidermal growth factor (HB-EGF) and cluster of differentiation 9 (CD 9) genes between diphtheria carriers and those infected with diphtheria. We searched the databases of the East Java Provincial Health Office and the Main Health Laboratory to identify diphtheria infection patients and carriers aged ≤18 years during 1 January 2012-30 August 2015. Each study participant was interviewed, had anthropometrical measurements obtained and blood was drawn for DNA sequencing of 2 genes coding for receptors and co-receptors of the diphtheria toxin, HB-EGF and CD9, and antibody titers against *Corynebacterium diphtheriae*. A total of 28 carriers and 97 patients with a history of diphtheria infection during the study period were included in the study. Silent mutations of codon 91 of exon 3 of the HB-EGF gene were found in 5 diphtheria carriers and 21 diphtheria cases, and of codons 171 and 173 of exon 6 of the CD9 gene in 1 carrier and 2 cases. We also found silent mutation of intron 5, position 35719 of the CD9 gene in 16 carriers and 39 cases. Statistical analysis showed no significant differences in the frequencies of mutations of exon 3 of the HB-EGF gene and exons 5 and 6 of the CD9 gene between carriers and cases. However, significantly more carriers had the mutation of intron 5 of the CD9 gene than cases. We concluded the genetic variability of the DT receptors in human was limited.

Keywords: diphtheria, carriers and patients, DNA sequence, HB-EGF and CD9 genes, Indonesia

INTRODUCTION

Diphtheria is a major public health problem in Indonesia. There has been a diphtheria outbreak in East Java Province, Indonesia, since 2011 (Husada *et al*, 2017). During 2011-2016, there were 3,353 reported diptheria cases; of which 69.4% were aged ≤18 years (Husada *et al*, 2017). Cases were reported throughout the province with a case fatality rate of 3.3% (Husada *et al*, 2017). Both cases and carriers were reported during the outbreak. Cases were

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those who had *Corynebacterium diphtheriae* and showed clinical signs and symptoms of diphtheria infection. Carriers had *C. diphtheriae* but did not have any clinical signs or symptoms. Carriers play a substantial role in disease transmission during outbreaks (Boschert *et al*, 2014; Williamson *et al*, 2016).

Several factors may be associated with diphtheria carrier state, such as genetics (King and Lively, 2012; Manry and Quintana-Murci, 2013). We aimed to look at the DNA sequences from two genes coding for the receptor and co-receptor of the diphtheria toxin (DT). Only toxigenic *C. diphtheriae* can cause disease; the main cause of morbidity is a potent toxin (Pappenheimer, 1977). Diphtheria toxin has 2 fragments, A and B. The B fragment is used for receptor attachment meanwhile A fragment will enter the cells and then destroy them (Unlu *et al*, 2003). To cause morbidity, the toxin must enter the cell through a process with four steps: attachment to a receptor, internalization into a vesicle, translocation of subunit A from an acidic vesicle to the cytosol, and finally ADP ribosylation of diphthamide (Middlebrook *et al*, 1978; Eidels *et al*, 1983). Many proteins and host genes contribute to each of those steps.

The cell receptor for the diphtheria toxin is pro-heparin binding-epidermal growth factor (pro-HB-EGF), a precursor of HB-EGF, which is a ligand of the EGF receptor (Naglich *et al*, 1992; Harris *et al*, 2003). There are three co-receptors, of which the most important is the cluster of differentiation 9 (CD9) (Mitamura *et al*, 1992). CD 9 does not bind DT directly. The effect of co-receptor like CD9 can enhance the receptor sensitivity until 25 times (Umata *et al*, 2000). Both HB-EGF and CD9 have physiological functions in the body other than their roles in DT pathophysiology (Umata *et al*, 2000). This strategy of *C. diphtheriae* to introduce the toxin into the cells is achieved by exploiting standard cellular components that are needed for routine physiological activities: the receptor (pro-HB-EGF), co-receptor (CD9), and some other important elements such as actin, furin, and H^+-ATP-ase. These are all common materials found in healthy cells (Saelinger, 2003).

Certain mammals, such as rats, are resistant to diphtheria toxin although the similar receptors are also present (Abraham *et al*, 1993). This is because the diphtheria toxin cannot attach to the cell receptor. There are 10 differences in amino acid composition for this receptor between rats and humans (Mitamura *et al*, 1995). The DNA sequence of HB-EGF genes in humans is different from the sequences in rats and mice. The role of the DNA sequence of the receptor and co-receptor regulating genes has been identified for some other diseases such as HIV infection (Hutter *et al*, 2009), but not for diphtheria.

Pro-HB-EGF and CD9 are controlled by HB-EGF and CD9 genes, respectively (Fen *et al*, 1993; Rubinstein *et al*, 1993). The HB-EGF gene, which is 14 kbp long, is on the 5q23 chromosome; this gene has 6 exons and 5 introns (Fen *et al*, 1993). The CD9 gene, which is 38 kbp long, is on the 12p13.31 chromosome; this gene has 8 exons and 7 introns (Rubinstein *et al*, 1993).

Lack of the literature regarding the role of genetic in diphtheria carriers and patients, the previous experience on human receptors in some diseases like HIV, and the fact that mice and rats are resistant to DT were our main reasons to conduct this study to look at the genetic variability of the diphtheria carriers and patients. We

focused on 2 primary receptor and co-receptor coding genes, HB-EGF and CD9. The objective of this study was to compare the DNA sequences of the HB-EGF and CD9 genes of the diphtheria carriers and patients with diphtheria infection. The results can improve our understanding regarding the pathogenesis of diphtheria infection.

MATERIALS AND METHODS

The participants of this study were all diphtheria carriers or patients with diphtheria infection (cases), aged ≤18 years, whose names were reported to the East Java Provincial Health Office and the Main Health Laboratory in Surabaya during 1 January 2012 - 30 August 2015. A case was identified if someone showed clinical signs and symptoms of diphtheria infection (mostly tonsillar and or pharyngeal diphtheria), and the cultures of the throat and nasal swab were positive for *C. diphtheriae*. Every time a case was seen, the health officer looked for the close contacts of that case. The health officers asked and checked the clinical signs or symptoms of diphtheria infection. The cultures from the throat and nasal swab were also taken. All contacts with *C. diphtheriae* but without any clinical signs and symptoms of diphtheria infection were categorized as carriers. Thus, the primary difference between diphtheria patients and carriers was clinical signs and symptoms of diphtheria infection. All microbiological cultures were performed during the initial identification. Because of the funding problem in East Java, there were no reculture attempts. Both carriers and patients were followed for at least 2 weeks. Treatments (mainly antibiotics and anti-diphtheria serum) were given to all diphtheria patients. For carriers, the health officers provided antibiotics (erythromycin) for 7-10 days.

Based on retrospective data, we prospectively visited all reported diphtheria patients and carriers. This activity lasted for 6 months until January 2016. Thus, there were 6 months – 2.5 years time difference between the initial identification of the diphtheria patients and carriers and the fieldwork for this study. For each participant, we conducted an interview, recorded anthropometric measurements, and obtained a blood sample. Collected data included age, sex, home district, housing situation, immunization status, and blood was obtained and examined for DNA sequencing and measurement of the anti-diphtheria antibody titer. The gene sequencing was conducted for the HB-EGF and CD9 genes.

For the HB-EGF gene, we focused on exon 3 which regulates code for 59 amino acids at the positions 75-133. We analyzed 380 base pair of DNA fragments. This exon controls the binding area of diphtheria toxin and the co-receptor CD9. For the CD9 gene, we focused on exons 5 and 6, which regulate coding for 33 and 30 amino acids, respectively. The amino acids build the binding site with pro-HB-EGF (also known as the EC2 loop). DNA was extracted using the Qiagen® DNA blood kit (Qiagen, Valencia, CA). The primers for exon 3 of the HB-EGF gene were: F 5′-CCTTTCAAGGACTATGCT-3′ and R 5′-CCCAACTTCCGCCCAGAGG-3′. The primers for the CD9 gene were: F 5′-GCCGTCTCTGCCCCTCTCTCG-3′ and R 5′-GAGATGGGTGCCCTGGGGCCC-3′. Data for the primers were obtained from the National Center for Biotechnology Information (NCBI) databases. The antibody titer examination was performed using the Vero cell method following the

Table 1
Characteristics of Diphtheria carriers and cases.

Characteristics	Participants (*N*=128)		*p*-value
	Carriers No. (%)	Cases No. (%)	
Age in years			
0-2	1 (3.7)	1 (1.0)	0.823[a]
>2-5	7 (25.9)	20 (19.8)	
>5-12	11 (40.7)	56 (55.4)	
>12-18	8 (29.7)	24 (23.8)	
Sex			
Boys	11 (40.7)	58 (57.4)	0.184[b]
Girls	16 (59.3)	43 (42.6)	
Parental ethnicity			
Madurese	12 (44.4)	56 (55.4)	0.423[b]
Others	15 (55.6)	45 (44.6)	
Maternal ethnicity			
Madurese	13 (48.1)	58 (57.4)	0.520[b]
Others	14 (51.9)	43 (42.6)	

[a]Mann-Whitney *U* test; [b]chi-square test.

standard for operating procedures manual from the Health Protection Agency, United Kingdom number R-6111/04-11 (2011) and a World Health Organization manual (WHO, 2013). The Vero cells came from The Dutch National Institute for Public Health and the Environment (RVIM), The Netherlands. The Vero cell assay is a cell micro-culture - neutralization test. We performed a titration of antitoxin in the serum, followed by spectrophotometry to find the equivalent point between the toxin and antitoxin (WHO, 2013).

We used the Statistical Program for the Social Sciences (SPSS) version 17 (IBM, Armonk, NY) to perform the Mann-Whitney *U* and chi-square tests. Ethical clearance was granted by Ethics Committee of Soetomo Hospital in Surabaya. Parents or guardian of the participants received the information about this study and signed the informed consent.

RESULTS

There were 127 diphtheria patients (cases) and 35 carriers, aged ≤18 years recorded in the database. Of the cases, 19 could not be located, 7 refused to participate, and 4 refused to give a blood sample. Of the carriers, 6 could not be located and 2 refused to participate. Therefore, 27 carriers and 97 cases from 21 districts were enrolled in the study. Of these, anti-diphtheria antibody titers were obtained in 25 carriers and 88 cases.

When comparing cases with carriers, the age and sex distribution and the ethnicity of the two groups were similar (Table 1). Most of the participants (77.3%) were aged ≥5 years.

In the HB-EGF gene, a silent mutation was found on codon 91 in 25 participants (5 carriers and 24 cases). In this mutation, the amino acid was histidine, and the

Table 2
Genotype distribution of the HB-EGF gene, codon 91, exon 3.

Genotypes	Carriers No. (%)	Cases No. (%)	Total No. (%)	*p*-value
TT	0 (0)	4 (4.1)	4 (3.2)	0.531[a]
CT	5 (18.5)	20 (20.6)	25 (20.2)	
CC	22 (81.5)	73 (75.3)	95 (76.6)	
Total (%)	27 (100)	97 (100)	124 (100)	

[a]Chi-square test for carriers and patients for 3 genotypes.

Table 3
Comparison of antibody levels by genotype for the HB-EGF gene, exon 3.

Genotypes (*n*)	Antibody levels (IU/ml)		
	Median	Minimum	Maximum
TT (3)	0.128	0	0.256
CT (24)	1.024	0	8.192
CC (86)	0.512	0	8.192

Kruskal-Wallis test: p=0.213; Two patients (1 homozygote and 1 heterozygote) did not have the antibody test examined.

wild-type nucleotide sequence was CAC. The mutation was the sequence CAT or the mixed sequence CAT/CAC (Table 2). The antibody levels are shown in Table 3. There were no significant differences between carriers and cases on the proportions with gene mutations, genotypes or antibody levels.

For HB-EGF exon 6, one case had a silent mutation of codon D171 (GAC changed to GAT), and 2 participants (1 carrier and 1 case) had a silent mutation of codon L173 (CTG changed to CTC). Fifty-five participants (16 carriers and 39 cases) had a mutation at intron 5 (between exon 5 and 6), at position 35719 of the CD9 gene. The mutation was a change from C to A. The mutant genotypes were significantly more common among the carriers. The prevalence ratio (PR) for mutant genotype was 2.133 (1.063-4.278). There were no significant differences in the antibody level between carriers and cases. Tables 4 and 5 show the proportions of carriers and cases with this mutation (C to A) and the antibody levels, respectively. Five participants had more than one mutation involving both genes. The median antibody level in this double mutation group was 0.512 International Units (IU)/ml.

DISCUSSION

Diphtheria carriers contribute to disease transmission (Boschert *et al*, 2014; Williamson *et al*, 2016). The duration of being a carrier is unclear; it could last for weeks until years (McCartney and Harvey, 1928; Miller *et al*, 1974; Begg and Balraj, 1995; Danilova *et al*, 2006). Carriers

Table 4
Proportions of carriers and cases by the CD9 gene intron genotype.

Genotypes	Carriers No. (%)	Cases No. (%)	Total No. (%)	*p*-value	PR (95%CI)
AA	9 (33.3)	14 (14.4)	23 (11.4)	0.041[a]	0.808[b]
AC	8 (29.6)	24 (24.7)	32 (33.0)		(0.660-0.989)
CC	10 (37.1)	59 (60.9)	69 (55.6)		
Total (%)	27 (100)	97 (100)	124 (100)		

[a]Chi-square test the proportion of 3 genotypes of carriers and patients; [b]PR between (AA and AC) *vs* CC; significant.

Table 5
Comparison of antibody levels based on the CD9 gene, intron 5 genotype.

Genotypes (*n*)	Antibody level (IU/ml)		
	Median	Minimum	Maximum
AA (23)	0.512	0	4.096
AC (32)	0.512	0	8.192
CC (58)	1.024	0	8.192

Kruskal-Wallis test, p=0.625; 7 mutated patients did not have the antibody level examination.

may have only partial immunity preventing the destruction of the microorganism (Casadevall and Pirofski, 2000). The difference between diphtheria carriers and patients is the ability of diphtheria toxin (DT) to penetrate and destroy cells. Both groups have toxigenic *C. diphtheriae*, but in carriers, the toxin does not damage the cells (Miller *et al*, 1974; Danilova *et al*, 2006; Unlu *et al*, 2013).

Most of our participants came from a part of the province dominated by the Madurese tribe/ethnicity. This area has more health-related problems than other areas, such as low immunization coverage. In the study area, most carriers and cases were ≤18 years. During a Russian diphtheria outbreak in the 1990s, most of the patients were adults (Neal and Efstratiou, 2007). However, many other reports from around the world have found most diphtheria outbreaks are comprised of children (Besa *et al*, 2014; Garib *et al*, 2015; Nanthavong *et al*, 2015).

All the mutations identified in this study were also found in the NCBI database, meaning they have been identified previously. However, the NCBI data was taken from a healthy population. Thus, there was no data on this mutation among diphtheria cases and carriers, neither the literature regarding the clinical impacts of those mutations. We believe the silent mutations identified in our study have no significant association with carriers or cases. The 4 amino acids coded for the HB-EGF gene, exon 3 are: Pheny 115, Leu 127, Ile 133, and His 135. The amino acid coded for an exon 4 is Glu 141 (Mitamura *et al*, 1997). The changes of these 4 amino

acids will change the susceptibility of the receptor to the DT (Cha *et al*, 1998). The HB-EGF gene exon 3 mutations seen in this study (cytosine to thymine) did not change the base group. Mutations on exon 6 of CD9 gene altered the purine into pyrimidine (guanine to cytosine).

The mutation identified in this study on the CD9 gene intron 5 was also found in the NCBI database. However, the impact of this mutation and its prevalence in the general population are unclear. Significantly more carriers than cases had the CD9 gene intron 5 mutation. Further study is needed to determine the importance of this mutation and its prevalences in different populations.

In most cases, silent mutations do not have a clinically important effect. However, some studies (Bhayal *et al*, 2015; Skevaki *et al*, 2015) have found differences in the risk for infection among people with some silent mutations or mutations in their introns. For examples, a gene mutation in the genes controlling TLR-2 and TLR-4 is associated with susceptibility to tuberculosis, leprosy, and pneumococcal infections (Manry and Quintana-Murci, 2013; Bhayal *et al*, 2015; Skevaki *et al*, 2015). Introns are conserved segments with an important unknown role. One study reported amino acid structure and function changes could occur in silent mutations caused by changes in transfer-RNA (Fernandez-Calero *et al*, 2016). Our study was not designed to evaluate this.

In conclusion, the DNA sequences for the HB-EGF gene, exon 3, and the CD9 gene, exons 5 and 6 were not significantly different between the diphtheria carriers and cases. However, the mutation of the CD9 gene intron 5 was significantly more common among diphtheria carriers than diphtheria cases. The clinical significance of this finding is unclear and requires further investigation.

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