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Phylogenetic relationship of Gram Negative Bacteria of *Enterobacteriaceae* Family in the Positive Widal Blood Cultures based on 16S rRNA Gene Sequences

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Abstract

The purpose of this study was to analyze the phylogenetic relationship of Gram negative bacteria (3 strains of *Salmonella typhi*, 1 strain of *Escherichia coli,* 1 strain of *Serratia marcescens*, and 3 strains of *Enterobacter cloacae*) of *Enterobacteriaceae* family in positive Widal blood cultures based on 16S rRNA gene sequences. The results respectively showed that each two 16S rRNA gene clones of *Serratia marcescens* KD 08.4 had a close relationship with 16S rRNA gene of *Serrratia marcescens* ATCC 13880 (similarity: 99.53-99.8%), *Eschericia coli* BA 30.1 with *Eschericia coli* ATCC 11775^T (similarity: 99.38-99.67%), *Salmonella typhi* BA 07.4, *Salmonella typhi* KD 30.4, and *Salmonella typhi* SA 02.2 with *Salmonella typhi* ATCC 19430^T (similarity: 99.4-100%) as well as the isolates of *Enterobacter cloacae* SA 02.1, *Enterobacter cloacae* BA 45.4.1, one 16S rRNA gene clone of *Enterobacter cloacae* TG 03.5 with *Enterobacter cloacae* ATCC 23373 (similarity: 99.0-99.87%).

Keywords: Widal, *Enterobacteriaceae*, 16S rRNA genes

Introduction

The typhoid fever incidence rate in Indonesia had reached 358-810/100.000 population/year with the mortality rate of 1-5% of patients (Anonymous, 2007). In Semarang, typhoid fever had been in the third rank of 10 major diseases after Dengue Fever and Diarrhea and gastroenteritis (Anonymous, 2008). Typhoid fever was a serious systemic infectious disease that was possibly accompanied by a variety of diseases such as dengue fever and malaria (Gasem *et al*., 2002).

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The clinical features of typhoid fever were unspecific that the gold standard diagnosis could not only depend on the clinical symptoms but it should also be supported by the laboratory diagnosis (Khoharo *et al*., 2010; Ley *et al*., 2010; Fadeel *et al*., 2011). The gold standard diagnosis of typhoid fever was by the finding of *Salmonella typhi* (*S. typhi*) in blood or bone marrow cultures (Khoharo *et al*., 2010; Ley *et al*., 2010). However, the facilities for culturing of bacteria were not always available, it was expensive, time consuming (seven days) and the result was frequently negative since the patients had consumed antibiotics. Widal test was a widely used laboratory test in Indonesia supporting the typhoid fever diagnosis as it was cheap, easy, fast, and simple. The sensitivity, specificity,

and predictive values of Widal test were various, due to the presence of anti-O and anti-H antibodies in patients infected by *Salmonella* sp., species of *Enterobacteriaceae* family member other than *Salmonella sp*. and malaria (Novianti, 2006; Beig *et al*., 2010). Darmawati *et al*., 2012, stated that there was bacterial species diversity of *Enterobacteriaceae* family members of such as *S. typhi, Serratia marcescens, Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae* in positive Widal blood cultures from Semarang. However, the phylogenetic relationship between species was unknown. Thus, the purpose of this study was to determine the phylogenetic relationship of Gram-negative bacillus bacteria of *Enterobacteriaceae* family members based on 16S rRNA gene sequences.

Materials And Methods

Bacterial Strains

There was a total of 8 isolates (3 isolates of *Salmonella typhi*, 1 isolate of *Escherichia coli*, 1 isolates of *Serratia marcescens*, and 3 isolates of *Enterobacter cloacae*) isolated from positive Widal blood samples of in and outpatients from Semarang (Tugurejo hospital, City Hospital of Semarang, Sultan Agung Islamic Hospital, Community Health Center of Bangetayu, and Community Health Center of Kedungmundu). The bacterial Identification used API 20E and API 50CHB/E media (Darmawati, *et al*., 2012).

DNA bacterial Extraction, PCR amplification, cloning, and DNA plasmid extraction with insertion and sequencing

DNA was extracted from eight bacterial strains used *DNeasy Blood & Tissue Kits* (Qiagen, K69504). The 16S rRNA gene amplification used *Applied Biosystems GeneAmp PCR System 2400*, 0.25µl *Takara Ex Taq,* 5µl 10X *Ex Taq* buffer, 4 µl dNTP Mixture (2.5 mM each), 2µl DNA template, 0.5µl primer 8F (1.0µM final conc.), and 0.5µl primer 4192R (1.0µM final conc.), 37.75µl sterile deionized water, for a total volume of 50µl. The thermal cyling was as follows: denaturation at 95^{o} C for 30 sec, annealing at 55^{o} C for 30 sec, extension at 72^{o} C for 1,5 min, and final extention 72^{o} C for 10 min for the total of 30 cycles. PCR products (1500bp) were visualilized through electrophoresis at 1% agarose gel with ethidium bromide added directly to the gel.

The amplified DNA bands were purified from agarose using glass powder method (Volgstein and Gillespie, 1979), ligated to T-Vector pMD20 (Takara Biotechnology), and transformated to *E.coli* DH5α. The plasmid DNA containing inserts was isolated, respectively amplified using primer M13 reverse, U515F, and M13-40 (Table 1). The amplified DNA were sequenced using primer M13 reverse, U515F, and M13-40. The DNA Sequencing was conducted with sequencer device of *ABI* Prism^{TM} 310 Genetic Analizer. The sequenced data were in the form of electrophenogram files and base DNA arrangement.

Tabel 1. Primer for 16S rRNA gene amplification dan sequencing

Primer	Sequences
8F	5′-AGA GTT TGA TCC TGG CTC AG-3′
1492 R	5′-AAG TCG TAA CAA GGT AAC C-3′
M13-RV	5′-CAG GAA ACA GCT ATG AC-3′
U515F	5′-GTG CCA GCA GCC GCG GTA A-3′
M13-40	5′- GTT TTC CCA GTC ACG AC-3′

Analysis and alignment of 16S rRNA gene sequences

The 16S rRNA sequence were analyzed and compared to the Gene Bank nucleotide database using Basic Local Alignment Search Tool (BLAST). The 16S rRNA Sequences of 8 bacterial strains were aligned using CLUSTAL X program.

Phylogenetic tree Construction

Phylogenetic tree was prepared using PHYLIP program, matrix similarity, and nucleotide difference of 16S rRNA between clones and strains analyzed with PHYDIT program.

Results And Discussion

The results of 16S rRNA gene amplification of 8 isolates of *Enterobacteriaceae* family members were shown in Figure 1 while the results of phylogenetic relationship analysis based on 16S rRNA gene sequences were shown in Figure 2. There was a total of 15 sequences with each isolate consisted of two 16S rRNA gene sequences derived from two clones of 16S rRNA gene sequences except TG 03.5 isolate and 5 sequences derived from 16S rRNA sequences of Gram-negative bacillus bacteria of Enterobacteriaceae family (Gene Bank, NCBI) consisting of *S. typhi* strain types of ATCC 19430^T (accession no. Z47544), *E. coli* 11775^T (X80725.1), *Ent. cloacae* ATCC 23373 (HQ651841.1), *Citrobacter freundii* ATCC 8090 (AJ233408.1), *Ser. marcescens* ATCC 13880 (AB594756.1). Two strains as out group used *Vibrio cholerae* ATCC 14547 (NR_044050.1) from Vibrionaceae family (negative catalase and positive oxidase) and *Pseudomonas aeruginosa* ATCC 23993 (FJ652615.1) from Pseudomonadaceae family (positive catalase and oxidase).

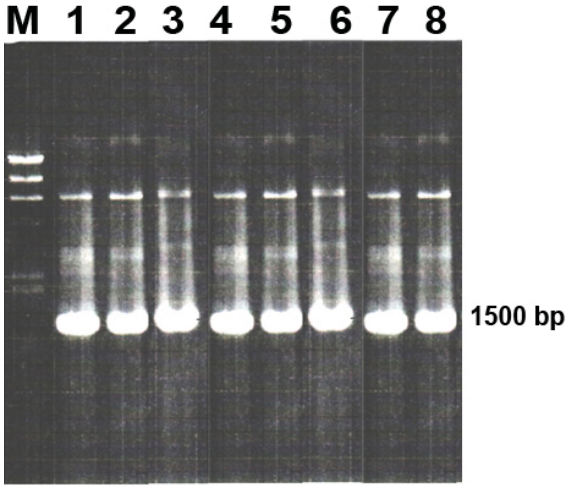

Figure 1. The results of 16S rRNA gene amplification of 8 bacterial isolates: (M) DNA Hind Marker III, (1) *S. typhi* SA 02.2, (2) *S. typhi* BA 07.4, (3) *S. typhi* KD 30.4, (4) *Ser. marcescens* KD 08.4, (5) *E. coli* BA 30.1, (6) *Ent. cloacae* SA 02.1, (7) *Ent. cloacae* TG 03.5, (8) *Ent. cloacae* BA 45.4.1

After the 16S rRNA gene sequences was aligned with Clustal-X program, the phylogenetic trees were arranged using PHYLIP program

The phylogenetic relationship analysis of 15 16S rRNA gene clones of 8 isolates (Figure 2) was divided into five clades. The first clade consisted of 6 16S rRNA gene clones derived from 3 isolates (SA 02.2, KD 30.4, and BA 07.4) and 2 reference strains: *S. typhi* ATCC 19430^T and *Ent. cloacae* ATCC 23373. The similarity value of those six clones with *S. typhi* ATCC 19430^T was 99.4 to 100% with the difference of 0-9 nucleotide, shown in Table 2.

Table 2. Matrix similarity and nucleotide difference of 16S rRNA gene sequences of Gram negative bacillus bacteria of Enterobacteriaceae family members on the first clade with reference strains of *S. typhi* ATCC 19430^T

Isolate Code	SA 022C3	SA 022C1	BA 074C1	KD 304C1	KD 304C3	BA 074C3	*S. typhi* ATCC 19430^T	*Ent. cloacae* ATCC 23373
SA022C3	---	4/1502	2/1503	2/1503	9/1500	5/1500	9/1503	27/1498
SA022C1	99.73	---	2/1502	2/1502	9/1499	5/1499	9/1502	27/1497
BA074C1	99.87	99.87	---	0/1503	7/1500	3/1500	7/1503	25/1498
KD304C1	99.87	99.87	100	---	7/1500	3/1500	7/1503	25/1498
KD304C3	99.4	99.4	99.53	99.53	---	6/1499	9/1500	28/1495
BA074C3	99.67	99.67	99.8	99.8	99.6	---	3/1500	22/1495
S. typhi ATCC 19430T	99.4	99.4	99.53	99.53	99.4	99.8	---	27/1504
Ent. cloacae ATCC23373	98.2	98.2	98.33	98.33	98.13	98.53	98.2	---

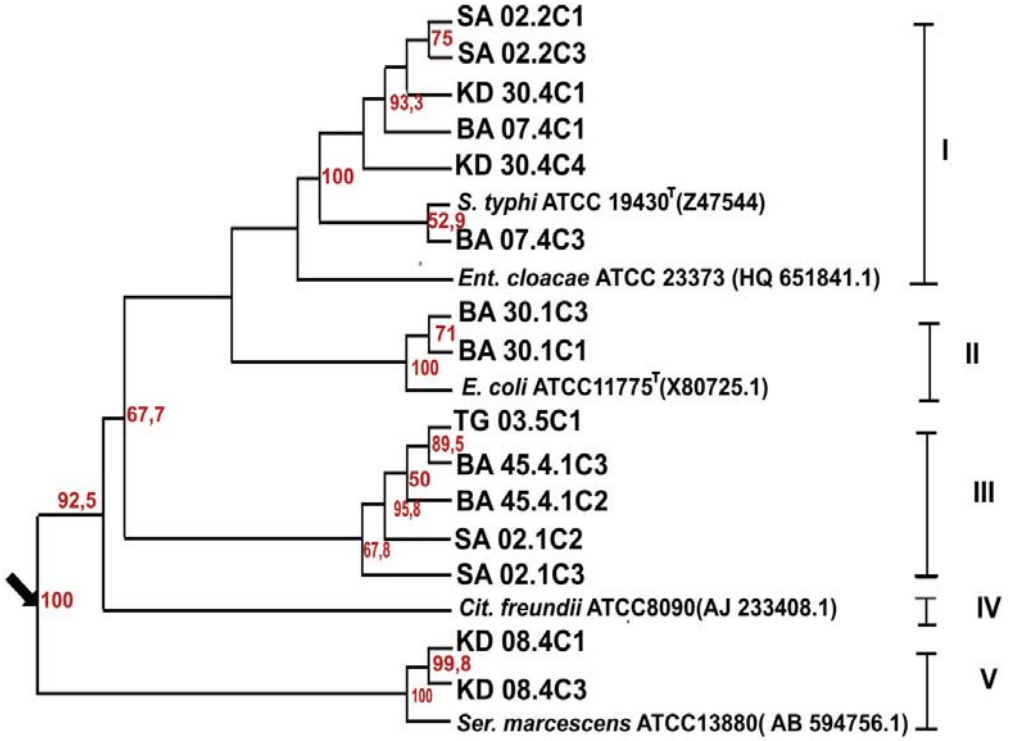

Figure 2. The phylogenic tree constructed based on Algoritme Neighbour Joining (Saitou and Nei, 1987) showing the kinship relationship between 8 isolates (15 clones of 16S rRNA) of Gram negative bacillus bacteria of Enterobacteriaceae family members, with 5 bacterial strains of Enterobacteriaceae members as reference based on 16S rRNA gene sequences. The root site determination was using outgroup of *Vibrio cholera* strain ATCC 14547 of Vibrionaceae family members and *Pseudomonas aeruginosa* strain ATCC 23943 of Pseudomonadaceae family members.

Based on the nucleotide similarity value, it could be concluded that isolate SA 02.2, KD 30.4, and BA 07.4 were identified as *S. typhi* members. Two different 16S rRNA clones, derived from one isolate also showed the presence of nucleotide differences of 3-7 (Similarity 99.53 to 99.80%).

The second clade consisted of two 16S rRNA gene clones derived from one isolate BA 30.1, with reference strain of *E. coli* ATCC 19430^T in the similarity value between 99.38 to 99.67% with nucleotide differences of 5-9 (Table 3). The isolate BA 30.1, based on its close relationship with the reference strain was identified as *E. coli* members.

The third clade consisted of five 16S rRNA gene clones derived from 3 isolates (BA 45.4.1, SA 02.1 and TG 03.5). The relationship between three bacterial isolates from Bangetayu Community health center, Tugurejo hospital and Sultan Agung Islamic Hospital showed the similarity value of 99.0 - 99.87% with nucleotide difference of 2-15 (Table 4), which had the closest kinship relationship with *Ent. cloacae* ATCC 23373.

Table 3. Matrix similarity and nucleotide difference of 16S rRNA gene sequences of Gram negative bacillus bacteria of Enterobacteriaceae family members on the second clade and reference strain of *E. coli* ATCC 19430^T

Isolate Code	BA 301C1	BA 301C3	*E. coli* ATCC11775T
BA 301C1	---	5/1503	8/1447
BA 301C3	99.67	---	9/1447
E. coli ATCC11775T	99.45	99.38	---

Table 4. Matrix similarity and nucleotide difference of of 16S rRNA gene sequences of Gram negative bacillus bacteria of Enterobacteriaceae family members on the third clade and reference strain of *Ent. cloacae* ATCC 23373

Isolate Code	BA 4541C3	TG 035C1	SA 021C2	BA 4541C2	SA 021C3	*Ent. cloacae* ATCC23373
BA4541C3	---	2/1500	7/1500	6/1499	12/1499	32/1495
TG035C1	99.87	---	5/1502	4/1501	10/1502	30/1498
SA021C2	99.53	99.67	---	9/1501	15/1501	31/1497
BA4541C2	99.6	99.73	99.4	---	12/1500	32/1496
SA021C3	99.2	99.33	99	99.2	---	23/1497
Ent. cloacae ATCC23373	97.86	98	97.93	97.86	98.46	---

The number of 16S rRNA gene copies in bacteria was various (1-15) in each genome. Each copy had the size approximately 1500 bp. Marchandin *et al*. (2003) reported that the 16S rRNA gene sequences in each copy of every organism were identical. The nucleotide difference of 16S rRNA gene copies was called micro-heterogeneity. The 16S rRNA gene sequences in 2 different 16S rRNA gene clones from the same isolates showed the presence of nucleotide difference with the similarity value of 99-100%. The results of this study was similar with the findings of Marchandin *et al*. (2003), that 4 16S rRNA gene copies in one bacterial strain of *Veillonilla* sp. ADV 360.1 showed two identical gene copies (similarity 100 %) and two various gene copies (similarity 98.5 to 99.8%).

The fourth clade consisted of one reference strain of *Citrobacter freundii* ATCC 8090. The fifth clade consisted of 2 16S rRNA gene clones derived from isolate KD 08.4 (similarity 99.53-99.8 %) and one reference strains of *Ser. marcescens* ATCC 13880 with nucleotide difference number of 3-7 nucleotides, were shown in Table 5.

Table 5. Matrix similarity and nucleotide difference of 16S rRNA gene sequences of Gram negative bacillus bacteria of *Enterobacteriaceae* family members on the fifth clade and reference strain of *Ser. marcescens* ATCC13880

Isolate Code	KD 084C3	KD 084C1	*Ser. marcescens* ATCC13880
KD084C3	---	3/1502	7/1488
KD084C1	99.8	---	6/1489
Ser. marcescens ATCC13880	99.53	99.6	---

Based on the nucleotide similarity value, it could be concluded that isolate KD 08.4 was identified as the isolate of *Ser. marcescens* KD 08.4 included as *Ser. marcescens* members.

The constructed phylogenetic trees based on Neighbor-Joining algorithm (Saitou and Nei, 1987) showed the relationship between 8 isolates of Gram-negative bacillus bacteria obtained from patients' positive Widal blood samples, included to Enterobacteriaceae family isolates which each consisted of 2 16S rRNA gene clones, except isolate TG 03.5, with 5 reference strains (*Ser. marcescens* ATCC 13880, *Cit. freundii* ATCC 8090 , *Ent. cloacae* ATCC 23373, *E. coli* ATCC 11775T, *S. typhi* ATCC 19430^T) based on 16S rRNA gene sequences. The data showed that the isolates found in positive Widal blood were *Ser. marcescens* KD 08.4, *E. coli* BA 30.1, *S. typhi* BA 07.4, *S. typhi* KD 30.4, *S. typhi* SA 02.2, and the isolates of *Ent. cloacae* SA 02.1, *Ent. cloacae* BA 45.4.1, *Ent. cloacae* TG 03.5 which had the closest kinship with *Ent. cloacae* ATCC 23373. The similarity value of those 8 isolates (15 16S rRNA gene clones) were between 97.86 to 100%, similar to what had been reported by Chang, *et al*. (1997).

Besides using numerical systematic based on phenotypic characters and chemical systematic, the bacterial identification could also conducted by using the molecular character systematic based on the nucleic acids, such as based on 16S rRNA genes. 16S rRNA genes were conserved genes which were present in all bacteria, that could be used to classify bacteria based on the kinship relationship. The genomic analysis was better than the protein analysis since it did not rely on certain genomic expression which encoded proteins and might result in phenotypic variation (Priest and Austin, 1995; Vandamme *et al*., 1996; Giammanco *et al*., 1999).

The results of phylogenic analysis based on 16S rRNA gene sequences showed that the isolates presented in positive Widal blood were *Ser. marcescens* KD 08.4 which had close relationship with *Ser. marcescens* ATCC 13880, *E. coli* BA 30.1 with *E. coli* ATCC 11775^T, *S. typhi* BA 07.4, *S. typhi* KD 30.4, and S. typhi SA 02.2 with S. typhi ATCC 19430^T, and the isolates of *Ent. cloacae* SA 02.1, *Ent. cloacae* BA 45.4.1, *Ent . cloacae* TG 03.5 with *Ent. cloacae* ATCC 23373. The similarity value of 8 isolates (15 16S rRNA gene clones) was between 97.86 to 100%. The Research conducted by Darmawati *et al*. (2013), showed that the classification based on biochemical characters was congruent with the results of classification based on total protein. This was also congruent with the classification based on 16S rRNA gene sequences. It might happen as the biochemical characteristics was the reflection of enzyme activities. The enzyme was functionally active protein as a result of translated expressed gene in a genome. Thus, the classification combining between the classification based on phenotypic, chemical, and molecular might result in accurate classification.

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Reference

Anonymous. 2007. Profile Health status of Province South Sulawesi, Indonesia. Ministry of of Health, Republic of Indonesia

Anonymous. 2008. Profil Kesehatan Kota Semarang 2008. Dinas Kesehatan. Jl. Pandanaran 79. Semarang

Beig, F. K., F. Ahmad, M. Ekram, I. Shukla., 2010. Typhidot M and Diazo test *vis-à-vis* blood culture and Widal test in the early diagnosis of typhoid fever in children in a resource poor setting. *Braz J. of Inf. Dis*, **14** (6): 589-593.

Chang, H. R., L. H. Loo, K. Jeyaseelan, L. Ernest and E. Stackebrandt., 1997. Phylogenetic Relationships of *Salmonella typhi* and *Salmonella typhimurium* Based on 16S rRNA Sequence Analysis. *Int. J. of Syst. Bacteriol,* **47** (4): 1253-1254

Darmawati, S. L. Sembiring, W. Asmara, W. T. Artama. 2012. Bacteria Species Diversity of Positive Widal Blood cultures from Semarang Based Phenotypic characters. Proceedings of the National Seminar on Biology of Sebelas Maret University of Surakarta.

Darmawati, S. L. Sembiring, W. Asmara, W. T. Artama, S. Syaiful. 2013. Chemosystematic of *Enterobacteriaceae* Familia Obtained from Blood Cultures Based on Total Protein Profiled. *Ind. J. of Biotechnol,* **18** (1): 58-63

Fadeel, M. A., B. L. House, M. M. Wasfy, J. D. Klena, E. E. Habashy, M. M. Said, M. A. Maksoud, B. A. Rahman & G. Pimentel., 2011. Evaluation of a newly developed ELISA against Widal, TUBEX-TF and Typhidot for typhoid fever surveillance. *J. of Inf. in Dev. Countries,* **5**(3): 169-175

Giammmanco, G., S. Pignato & G. M. Giammanco., 1999. Recent trends in Salmonellosis epidemiology. *J. of Prevent. Med. and Hygiene,* **40**: 19-24

Khoharo, H. K., S. Ansari, F. Qureshi., 2010. Evaluating Single Acute-phase Widal test for the diagnosis of Typhoid Fever. *Medical Channel*, **16** (1): 42-44

Ley, B., G. Mtove, K. Thriemer, B. Amos, L. Seidlein, I. Hendriksen. A. Mwambuli, A. Shoo, R. Malahiyo S. M. Ame, D. R. Kim, L. R. Ochial, J. D. Clemens, H. Reyburn, H. Wilfing, S. Magesa & J. L. Deen., 2010. Evaluation of the Widal tube agglutination test for the diagnosis of typhoid fever among children admitted to a rural hospital in Tanzania and a comparison with previous studies. *Bio Med Central Infectious Diseases,* **10**:180

Marchandin, H., C. Teyssier, M. S. de Buochberg, H. Jean-Pierre, C. Carriere & E. Jumas-Bilak., 2003. Intra-chromosomal heterogeneity between the four 16S rRNA gene copies in the genus Veillonella: implications for phylogeny and taxonomy

Novianti, T., 2006. Examination of *Anti-Salmonella typhi* IgM for Typhoid Fever Diagnosis Laboratory Information. ISSN 0854-7165. No. 5/2006.

Priest, F. and B. Austin. 1993**.** *Modern Bacterial Taxonomy*. Second edition. Chapman and hall. London

Saitou, N. and M. Nei., 1987. The neighbor-joining method: a new method for Reconstructing phylogenetic trees. *Mol Biol Evol,* **4** (4): 406-425

Vandamme, P., B. P. M. Gillis, P. De Vos, K. Kerstres and J. Swings., 1996. Polyphasic Taxonomy, a consensus approach to bacterial systematic. *Microbiol Rev,* **60** (2): 407-438

Volgstein, B., and D. Gillespie., 1979. Preparative and analytical purification ofDNA from agarose. *Proc. Natl. Acad. Sci*. U.S.A. **76**:615-619.