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Study of *Streptococcus pneumoniae* serotype 19A blood isolates from Thailand by enterobacterial repetitive intergenic consensus (ERIC)-PCR typing

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ABSTRACT: *Streptococcus pneumoniae* serotype 19A is frequently isolated worldwide. In this study, the clonal relationships among 62 isolates from different patients from 21 hospitals between 2008 and 2018 were characterized using enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR). The different band patterns that appeared upon agarose gel electrophoresis were used to construct an unweighted pair group method with an arithmetic mean (UPGMA) dendrogram. There were 23 different ERIC types (E1–E23). The most prevalent type was E9, accounting for 19.36% of all isolates, followed by E6 at 16.13%, E5 at 11.30%, and 13 ERIC types present in only one isolate or at 1.61% each. Using an additional study to determine the clonal relationships, we compared our ERIC-PCR results to the corresponding multilocus sequence types (MLSTs) from our recent study with the same 62 *S. pneumoniae* serotype 19A isolates. The results showed there were 20 different MLST types and that ERIC-PCR was comparable to MLST as a valuable complementary tool for the investigation of *S. pneumoniae* serotype 19A isolates. Furthermore, ERIC-PCR is very fast, affordable, and easy to perform compared to MLST. However, there is less concordance between these two methods. These results suggest a high diversity of different ERIC-PCR and MLST patterns. Overall, the combination of results from both methods can add greater discrimination and complementary information for the differentiation of *S. pneumoniae* strains in Thailand.

KEYWORDS: *Streptococcus pneumoniae*, serotype19A, ERIC-PCR, multilocus sequence typing

INTRODUCTION

Streptococcus pneumoniae is a highly virulent pathogen that can cause pneumonia, bacteremia, sepsis, and meningitis. It causes millions of deaths worldwide and has a significant morbidity rate, particularly in the elderly and young children [1]. Invasive pneumococcal disease (IPD) is when *S. pneumoniae* invades sterile sites, such as blood, pleural fluid, cerebrospinal fluid, joint fluid, tissues, and organs [1, 2]. The Centers for Disease Control and Prevention (CDC) in the US has reported an annual incidence of IPD of 10.6/100,000 people. The CDC noted that IPD cases occur more in adults than in children, and that bacteremia was present in 20% of total IPD cases [3]. In Latin America and the Caribbean, *S. pneumoniae* causes 12,000–18,000 deaths, along with 4000, 1229, and 327,000 cases of meningitis, sepsis, and pneumonia yearly and involves patients < 5 years of age [4]. At present, 98 serotypes of *S. pneumoniae* have been reported, depending on the polysaccharide composition of its bacterial capsule, which is the most important pneumococcal virulence factor of its antiphagocytic activity [5]. The decline in IPD found in one study, following the introduction of the 7-valent pneumococcal conjugate vaccination (PCV7), was tempered by the emergence of non-vaccine serotypes, particularly 19A [6]. Serotype 19A has been a subject of concern in some regions since PCV7 implementation is due to increased prevalence and drug resistance [7]. However, a previous report suggested that in addition to the vaccine, areas where PCV7 was unattainable or was scarcely used also showed an increase in *S. pneumoniae* serotype 19A prior to PCV7 implementation [8]. The recognition that serotype 19A is a predominant serotype associated with IPD and that *S. pneumoniae* serotype 19A clinical isolates have high rates of multiple drug resistance makes *S. pneumoniae* serotype 19A interesting in the field of epidemiology and of great clinical importance [9]. Furthermore, the increase in serotype 19A IPD in several countries following the implementation of PCV-7 or PCV-10 has made it the most common serotype identified in recent years [10], and this highlights the need for the continued surveillance of serotype 19A [9].

Several genetic characterizations or molecular typing can be used to discriminate different isolates of *S. pneumoniae* within the same serotypes, such as pulsed-field gel electrophoresis, restriction fragment length polymorphism, amplified fragment length polymorphism, penicillin binding protein fingerprinting, and MLST [10]. The principle of MLST is the system-

atic DNA sequencing of 7 well-conserved, housekeeping genes within a bacterial genome to characterize the isolates [11]. ERIC-PCR is a simple, sharp, and cost-effective genotyping technology that discriminates different types of bacterial isolates. The widespread distribution of these repetitive DNA elements of various bacteria should enable the rapid identification of bacterial species and isolates [12–15], which is useful for the analysis of bacterial genomes [16–19]. The ERIC sequences can be utilized as efficient primer binding sites in a PCR to produce fingerprints of different bacterial genomes [20–22].

The aim of the present study was to study genetic characterizations by determining ERIC types of *S. pneumoniae* serotype 19A isolated from the blood of patients. We were also interested in comparing ERIC types with MLST types from our recent report [11] as an extension of our previous work. Though MLST is an appropriate method to determine clonal relationships, it is more expensive than ERIC-PCR.

MATERIALS AND METHODS

Bacterial isolates

The study protocol was approved by the Siriraj Institutional Review Board (SIRB with reference code EC 002052). We used 62 isolates of *S. pneumoniae* serotype 19A collected from 21 hospitals in Thailand from 2008–2018 in our previous study [11]. *S. pneumoniae* was isolated from hemoculture and identified according to standard microbiological methods [23]. Serotypes of *S. pneumoniae* 19A were identified by sequential multiplex PCR [24]. Each isolate was kept at −80 °C in 5% tryptic soy broth with 20% glycerol (v/v) until use.

Molecular typing of *S. pneumoniae* serotype 19A

DNA from *S. pneumoniae* colonies grown overnight at 35 °C on blood agar containing 5% sheep blood were extracted using the Gentra Puregene Yeast/Bacteria kit (QIAGEN). DNA was used as a template for polymerase chain reactions. In molecular typing to study the genetic relationships among *S. pneumoniae* serotype 19A isolates, ERIC-PCR was performed using the primers (ERIC1R, ATGTAAGCTCCTGGGGATTCAC; ERIC2, AAGTAAGTGACTGGGGTGAGCG; random amplicon sizes) and the PCR conditions previously described [12]. Briefly, PCR was carried out in a total volume of 20 μl. The reaction mixture contained the following reagents: 1× of 10× buffer, 0.2 mM dNTPs, 0.4 μM ERIC1R and 0.4 μM ERIC2 primers, 1U of Taq DNA polymerase, and 2 μl of genomic DNA. The PCR cycles included initial denaturation at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 49 °C for 18 s, extension at 72 °C for 3 min, and a final extension at 72 °C for 10 min.

The PCR products were analyzed by gel electrophoresis on 1.5% agarose gel in 1× Tris-Borate-EDTA (TBE) buffer. After loading the product, the gel was run at 77 V for 45 min. Then, the gel was stained with ethidium bromide and band images were determined under UV light using a gel documentation system machine (Syngene, UK). The band pattern was analyzed and a UPGMA dendrogram was constructed using the CLIQS program (Tatallab Ltd., England). This program provides a similarity matrix and creates a clustering of the ERIC-PCR results.

Data analysis

Data was analyzed with IBM SPSS Statistics version 20.0 (IBM Corp., Armonk, NY, USA) for descriptive analyses. Categorical variables were expressed as a percentage, mean, standard deviation, or range.

RESULTS

In this study, the patients' ages ranged from 9 months to 87 years, with a mean age and standard deviation of 36.08 ± 33.86 years. Of the 62 patients, the ratio of male:female was 33:29 (1.14:1). The source of clinical specimens was blood samples. The results from the molecular typing to determine the genetic relationships of all *S. pneumoniae* serotype 19A isolates using the ERIC-PCR technique (Fig. 1) demonstrated various DNA fingerprints. The differences in the band patterns that appeared on agarose gel were used to construct a UPGMA dendrogram (Fig. 2). The results showed that there were 23 different ERIC types (E1–E23) at the 85% cutoff for a similar value (red line). The most prevalent type was ERIC type E9, accounting for 19.36% of all the isolates, followed by ERIC type E6 at 16.13%, and ERIC type E5 at 11.30%, while 13 ERIC types were present in only one isolate at 1.61% each.

Correlation between MLST and ERIC-PCR

The correlation between the MLST and ERIC methods is shown in Table 1. The results show that only 4.8% (3/62) of sequence types (STs) were identical with the STs from our previous study [11] and ERIC types from this study, i.e., ST14390 = ERIC type E21, ST2062 = ERIC type E22, and ST14389 = ERIC type E23. However, several ERIC types and STs did not match, such as ST320 ($n = 25$) from our previous study [11], which belonged to 9 ERIC types [ERIC type E2 ($n = 1$), ERIC type E5 ($n = 5$), ERIC type E6 ($n = 6$), ERIC type E7 ($n = 1$), ERIC type E8 ($n = 1$), ERIC type E9 ($n = 8$), ERIC type E15 ($n = 1$), ERIC type E16 ($n = 1$), and ERIC type E19 ($n = 1$)] in this study. Another example was ERIC type E9 in Table 1, which belonged to 5 STs (ST63, ST320, ST8346, ST10923, ST144151). Thus, there was less concordance between ERIC and MLST in this study.

Discriminatory index analysis is one tool to assess probability that two unrelated isolates from the test population could be assigned to different typing groups

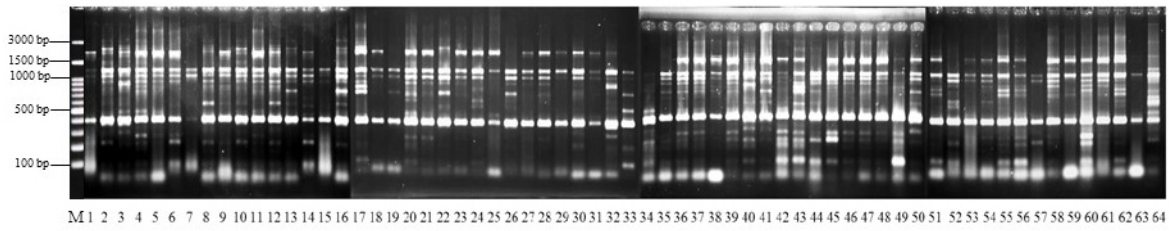

Fig. 1 ERIC-PCR pattern on gel electrophoresis. Lanes 1–62: *S. pneumoniae* serotype 19A, lane 63: *S. pneumoniae* serotype 14, lane 64: other species (*Neisseria gonorrhoeae*).

Table 1 ERIC types (E) and sequence types (ST) distribution of invasive pneumococcal serotype 19A.

ERIC types (no. of isolates)	% (total = 62)	Multilocus sequence types MLST-ST* (no. of isolates)
E1 (1)	1.61	ST63 (1)
E2 (1)	1.61	ST320 (1)
E3 (1)	1.61	ST2930 (1)
E4 (3)	4.84	ST2930 (3)
E5 (7)	11.30	ST320 (5), ST63 (1), ST4467 (1)
E6 (10)	16.13	ST95 (2), ST230 (1), ST320 (6), ST14414(1)
E7 (3)	4.84	ST320 (1), ST2930 (1), ST12360 (1)
E8 (3)	4.84	ST230 (2), ST320 (1)
E9 (12)	19.36	ST63 (1), ST320 (8), ST8346 (1), ST10923 (1), ST14415 (1)
E10 (2)	3.23	ST1701(1), ST14413(1)
E11 (1)	1.61	ST8346 (1)
E12 (5)	8.07	ST230 (2), ST2930 (2), ST14391 (1)
E13 (2)	3.23	ST230 (1), ST14392 (1)
E14 (2)	3.23	ST4901 (1), ST10379 (1)
E15 (1)	1.61	ST320 (1)
E16 (1)	1.61	ST320 (1)
E17 (1)	1.61	ST2930 (1)
E18 (1)	1.61	ST2930 (1)
E19 (1)	1.61	ST320 (1)
E20 (1)	1.61	ST230 (1)
E21 (1)	1.61	ST14390 (1)
E22 (1)	1.61	ST2062 (1)
E23 (1)	1.61	ST14389 (1)

* Data from our recent report [11], which indicated there were 20 different MLSTs.

or clusters. It was calculated as per the Hunter-Gaston formula [25] as follows:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} x_j(x_j - 1)$$

D represents the index of discriminatory power, N the number of unrelated strains tested, S the number of different types, and x_j the number of strains belonging to the j-th type.

In this study, the discriminatory index (D) of ERIC-PCR was 0.9186, whereas the D of MLST from our previous study was 0.8086.

DISCUSSION

In a prior systematic review, the prevalence of *S. pneumoniae* serotype 19A was high, with no observed changes in the trend [26]. In the same study, there was a reduction (19.9%) in the total number of IPD isolates (probably due to the usage of PCVs [27]), whereas the proportion of *S. pneumoniae* serotype 19A compared to the total number of IPD isolates was double.

The advantages of pulsed-field gel electrophoresis, restriction fragment length polymorphism, amplified fragment length polymorphism, penicillin binding protein fingerprinting when compared with ERIC in this study, are that the obtained profiles make excellent epidemiological markers and are used extensively in tracing the spread of pneumococcal isolates. Their disadvantages when compared with ERIC is the cumbersome process, rapid and discriminatory typing, but that isn't the case for population structure analysis [28].

We used the ERIC-PCR technique to track the spread of *S. pneumoniae* serotype 19A. This technique is fast and inexpensive compared to MLST or pulsed-field gel electrophoresis. It is also reliable for epi-

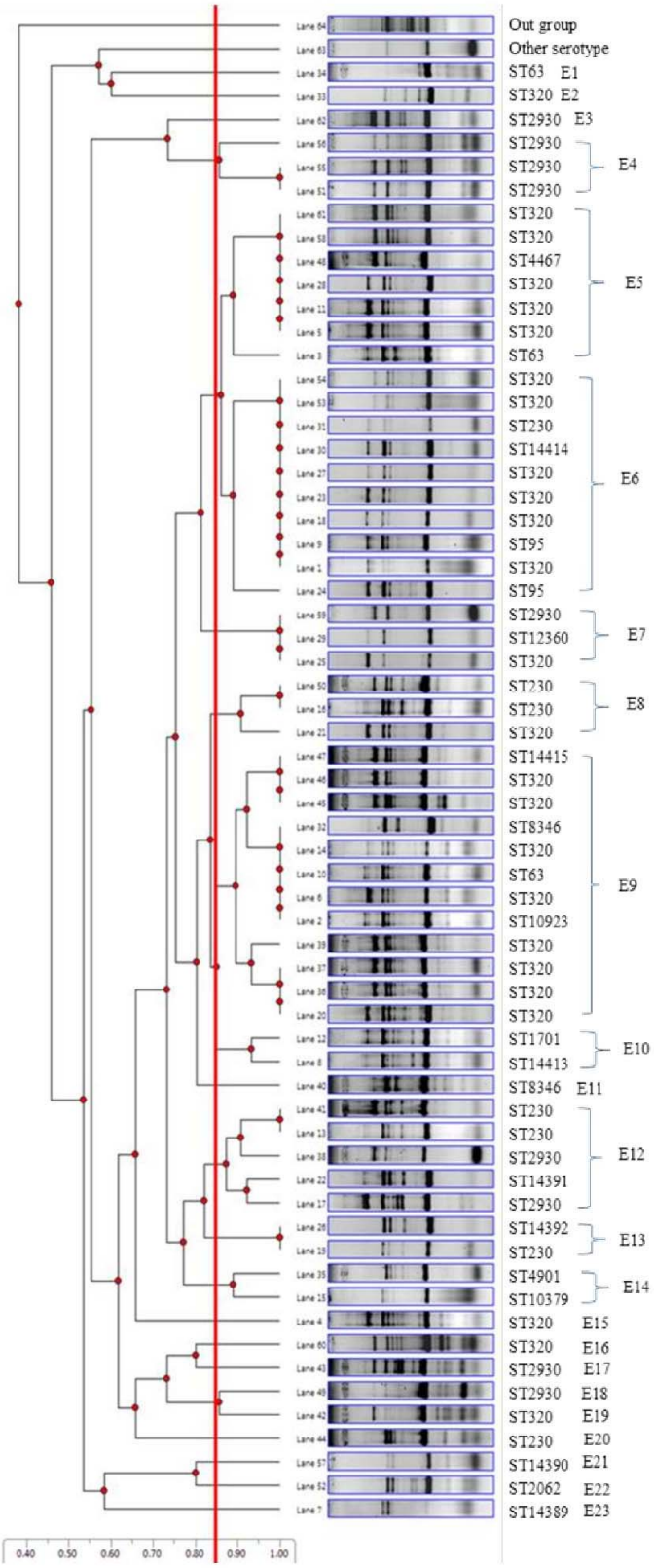

Fig. 2 UPGMA dendrogram. Lanes 1–62: *S. pneumoniae* serotype19A, lane 63: *S. pneumoniae* serotype 14, lane 64: other species (*Neisseria gonorrhoeae*).

demiological studies. The evaluation of ERIC-PCR as a tool for molecular analysis of invasive *S. pneumoniae* serotype 19A revealed 23 different patterns of DNA fragments, based on the locations of specific repetitive sequences in the pneumococcal genomes. MLST, which is based on the nucleotide polymorphism of DNA sequences of 7 housekeeping genes, revealed 20 STs [11]. A dendrogram is a branching diagram that represents similar relationships among a group of entities. This infers that a variation between the two techniques would be observed. Even though some ERIC patterns showed concordance with MLST results, we also found inordinate discrimination for some STs, such as ST2930, ST230, and ST320. In addition, genetic variations may interfere in different typing methods. As a consequence, the results would unlikely have 100% concordance [14]. In general, ERIC-PCR indicates a profile of DNA bands of different sizes, which depend on the various locations of specific repetitive sequences on the whole bacterial genome. On the contrary, MLST is concerned only with the DNA sequences of 7 specific housekeeping genes. While MLST gives excellent information on the genetics of the housekeeping genes, it provides little knowledge on specific genetic changes beyond the targeted loci. Therefore, the clonality of *S. pneumoniae* serotype 19A isolates may be overestimated by ERIC-PCR compared to MLST. Therefore, choosing ERIC-PCR as the typing method should be of concern.

In this study, the ERIC-PCR technique, using two-ERIC primer sequences, was first applied to *S. pneumoniae* serotype 19A and it showed pretty good results. The presence of ST320, ST230 in all ERIC patterns may be due to the fact that both STs were predominant in our recent study with the same 62 *S. pneumoniae* serotype 19A isolates [ST320 = 25/62 (60%) being the most predominant ST; ST230 = 7/62(12%)] [11]. Among the 62 isolates tested, we found at least 4 pairs of strains that had closely-related band patterns, which belonged to the same year of isolation. Moreover, this method is one of the fastest molecular typing techniques [12] and is cheap compared to MLST. Thus, to roughly study the genetic relationship of *S. pneumoniae* with a limited budget and time constraints along with an uncomplicated procedure, this method may provide an option as a molecular typing tool. Currently, there are several studies that have applied this method for genotypic characterization with promising results, such as for *Acinetobacter baumannii* [12, 15], *Corynebacterium pseudotuberculosis* [16], *Escherichia coli* [17], *Haemophilus parasuis* [18], *Pasteurella multocida* [19], *Pseudomonas aeruginosa* [14], *Shigella* [20], *Vibrio parahaemolyticus* [21], and viridans streptococci [22]. There was only one report which used the ERIC method for different clonalities of *S. pneumoniae* [29]. In that study, PCR fingerprinting with a single primer homologous to ERIC2 was used. They studied 28 pneumococcal isolates from blood and/or cerebrospinal fluid of 21 patients. Their results cannot be compared with this study as we used two ERIC primers.

The discriminatory power (D) of ERIC-PCR in this study was 0.9186 and correlated with 23 ERIC-PCR types, whereas the D of MLST was 0.8086 and it correlated with 20 MLST types. ERIC-PCR, MLST, and PFGE provide measures of genetic diversity, but they are not equivalent in principle. Several factors must be considered when selecting a molecular typing strategy. These include discriminatory power, reproducibility, and typeability, as well as the biological basis for grouping similar strains and cost. ERIC-PCR is a rapid, inexpensive and easily performed PCR-based typing technique that can be used to screen genetic relatedness, especially in a resource-limited laboratory.

In conclusion, our results suggest a high diversity of different ERIC-PCR and MLST patterns. The combination of results from both methods can provide greater discrimination and complementary information for the differentiation of *S. pneumoniae* strains in Thailand.

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