INVASIVE *STREPTOCOCCUS PNEUMONIAE* SEROTYPE 19A IN THAILAND (2008-2018)

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Abstract. Following introduction of a 7-valent pneumococcal polysaccharide-protein conjugate vaccine (PCV7), incidences of invasive pneumococcal disease caused by *Streptococcus pneumoniae* serotype 19A non-susceptible penicillin dramatically increase worldwide. In Thailand, as molecular characterization of invasive *S. pneumoniae* serotype 19A is sparse, we determined multilocus sequence types (MLSTs) and antibiogram profile of invasive disease serotype 19A isolated from 21 hospitals during 2008-2018. *S. pneumoniae* (n = 62) demonstrated twenty different STs, grouped into three main clonal complexes (CCs), namely, CC63 (36%), CC230 (21%) and CC320 (43%), with the most predominant MLST being ST320 (n = 25), followed by ST2930 (n = 9), ST230 (n = 7), ST63 (n = 3), ST95 and ST8346 (n = 2 each); there were also seven isolates having novel STs (14415, 14391, 14392, 14389, 14390, 14413, and 14414). Based on criteria for meningitis, 93 and 52% of isolates were non-susceptible to penicillin and cefotaxime/ceftriaxone, respectively while 30 and 8% of non-meningitis isolates were non-susceptible, respectively. Non-susceptibility to meropenem and erythromycin constituted 72% of the isolates while 100% were susceptible to levofloxacin, ofloxacin and vancomycin. The predominant ST320 and ST2930 isolates were multidrug resistant (*ie*, resistant to at least three classes of antimicrobial) and were disseminated among the study hospitals during the survey period. Hence, control and prevention of infection by highly antimicrobial-resistant *S. pneumoniae* serotype 19A should be a major health care concern.

Keywords: *Streptococcus pneumoniae* serotype 19A, antibiogram profile, clonal cluster, multilocus sequence type, Thailand

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INTRODUCTION

Streptococcus pneumoniae is a virulent bacterial pathogen and a leading cause of sepsis, meningitis and community-acquired pneumonia especially in children, the elderly and immunocompromised patients (Srifuengfung *et al*, 2014). Invasive pneumococcal disease (IPD) refers to pneumococci that have already invaded normally sterile sites (blood, cerebrospinal fluid, joint fluid, pericardial fluid, or pleural fluid) (Chiu *et al*, 2017). In USA, the annual incidence of IPD is 10.6/100,000 population, with more IPD cases in adults than children, and bacteremia is present in 20% of all cases (Berical *et al*, 2016). In Latin America, annually there are approximately 327,000 cases of *S. pneumoniae*-associated pneumonia in children, resulting in 12,000-18,000 deaths (Hawkins *et al*, 2017).

At present, there are 98 immunologically distinct serotypes of pneumococci, each varying in polysaccharide structure of the capsule (Paton and Trappetti, 2019), which protects pneumococci from phagocytosis and is a virulence factor (Geno *et al*, 2015). A seven-valent pneumococcal-diphtheria CRM197 protein conjugate vaccine (PCV7), consisting of seven serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F), which constitute the most common causes of IPD in USA and confer drug-resistance in children was introduced in 2000 (Geno *et al*, 2015). However, following adoption of PCV7 vaccine, IPD-causing *S. pneumoniae* serotype 19A, previously a rare etiology of the disease (Hanage, 2007), showing multidrug resistance (MDR, *ie*, resistant to at least three antimicrobial classes) and carrying a multilocus sequence type 320 (ST320) was reported in Germany (van der Linden *et al*, 2013) and USA (Hulten *et al*, 2013; Rudolph *et al*, 2013). Introduction of PCV13 vaccine (containing serotypes originally present in PCV7 plus serotypes 1, 3, 5, 6A, 7F and 19A) resulted in a decline of the pathogenic *S. pneumoniae* serotype 19A in USA (Hulten *et al*, 2013). In Norway where antimicrobial usage is strictly regulated,penicillin-susceptible *S. pneumoniae* ST199 strain is predominant in the post-PCV7 era (Vestrheim *et al*, 2012). A study from the Asian Network for Surveillance of Resistant Pathogens in 2011 reported ten *S. pneumoniae* isolates from Thailand belonging to clonal complex CC172 (n = 4), ST878, ST2636, ST4458 (n = 1 each), ST4826 (n = 2), and ST6002 (n = 1), and in Malaysia, CC172 (n = 3), CC320 (n = 3), ST3781 (n = 2), ST63, ST4458, ST2636, ST2855, ST4937, ST6003 (n = 1 each) was reported (Shin *et al*, 2011).

Here, we report the genetic characteristics and antibiogram of IPD *S. pneumoniae* serotype 19A in Thailand during 2008-2018. The findings should provide important and valuable information about molecular epidemiology and drug susceptibility

of this serotype.

MATERIALS AND METHODS

Bacterial isolates source

Invasive *S. pneumoniae* infection isolates collected during 2008-2018 were kindly provided by the "Pneumococcal Laboratory Network" (Bhumibol Adulyadej Hospital, Phramongkutklao Hospital, and Ramathibodi Hospital and Siriraj Hospitals) and 17 other alliance hospitals (9 private and 8 public hospitals) located in Bangkok (*n* = 62 isolates). Non-duplicate isolates were confirmed to be *S. pneumoniae* by colony morphology, alpha-hemolysis on sheep blood agar, Gram staining, and optochin susceptibility and bile solubility tests (Spellerberg and Brandt, 2015), then stored in tryptic soy broth (TSB) (Oxoid, Hampshire, United Kingdom), containing 20% glycerol at -80°C until use.

Research protocols were approved by Siriraj Institutional Review Board (SIRB reference no. EC 002052).

Serotype 19A determination

Serotyping was identified by PCR as previously described (Pai *et al*, 2006). In brief, pneumococcal isolates were subcultured on sheep blood agar (Clinag Co Ltd, Bangkok, Thailand) overnight at 35°C under an atmosphere of 5% CO_2 and DNA was extracted from a loopful of pneumococci culture using a boiling method. DNA concentration was measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc, Wilmington, DE) and stored at -20°C until use. PCR mixture (20 µl) contained 10 µl of 2X KAPA2G Fast Multiplex Mix (Sigma-Aldrich, Burlington, MA), 3 pmol each of 19A Forward primer (5'-GTTAGTCCTGTTTTAGATTTAT-TTGGTGATGT-3') and 19A Reverse primer (5'-GAGCAGTCAATAAGAT-GAGACGATAGTTAG-3'), 1 µl of DNA and Milli Q water (to make up to reaction volume). Thermocycling was performed in a Gene Amp PCR system 2400 thermal cycler (Perkin-Elmer, Waltham, MA) as follows: 95°C for 3 minutes; 30 cycles of 95°C for 30 seconds, 54°C for 40 seconds and 72°C for 60 seconds. Amplicon of *cps*19aK (478 bp) was analyzed by 1% agarose gel-electrophoresis and ethidium bromide staining. Controls were *S. pneumoniae* of known serotypes (1, 3, 4, 5, 6, 7F/A, 7B/C, 8, 9V/A, 9N/L, 10A, 11A/D, 12F/A, 14, 15A, 15B/C, 16, 17F, 18, 19A, 19B/C, 19F, 20, 21, 22F, 23A, 23B, 23F, 24, 25A/F/38, and 35B. All known serotypes are kindly provided from Microbiology Division, Department of Medical Science, Ministry of Public Health, Thailand. In each PCR tube, primers *cps*A-forward primers (5' GCA GTA CAG CAG TTT GTT GGA CTG ACC 3') and *cps*A-reverse primers (5' GAA TAT TTT CAT TAT CAG TCC CAG TC 3') targeting pneumococcal *cpsA* were included as internal control. Names and sequences of all known serotypes were previously reported by Pai *et al* (2006).

Multi-locus sequence typing (MLST) protocol

DNA from *S. pneumoniae* serotype 19A isolates were extracted using Puregene Yeast/Bacteria kit (QIAGEN, Omega Bio-Tek Inc, Norcross, GA). A set of seven housekeeping genes (*aroE*, *ddl*, *gdh*, *gki*, *recP*, *spi*, and *xpt*) were amplified using primers listed in Table 1 (Enright and Spratt, 1998). The PCRs were carried out in a total volume of 50 µl. The reaction mixture contained the following reagents: 38 µl of PCR grade water, 5 µl of 10x buffer, 1 µl of 10 mM of dNTPs, 1 µl of 20 µM of forward and reverse primer, 1 µl of DNA Taq polymerase and 4 µl of genomic DNA. The PCR cycles included initial denature at 94°C for 5 minutes, 10 cycles of denature at 94°C for 15 seconds, anneal at 54°C for 30 seconds, extension at 72°C for 45 seconds, followed by 20 cycles of the same denature and anneal protocol but extension at 72°C for 55 seconds, then final extension has been done at 94°C for 10 minutes. Amplicons were analyzed by 1% agarose gel-electrophoresis and ethidium bromide staining. Each amplicon was purified from excised gel band using a Gel and PCR Clean-up Kit (NucleoSpin®, Takara Bio Inc, Kusatsu, Shiga Prefecture, Japan) and subjected to nucleotide sequencing (Ward Medic Ltd, Bangkok, Thailand). A Molecular Evolutionary Genetics Analysis version 7 (MEGA 7) software was used to align and trim the sequences (Kumar *et al*, 2016), which were submitted to the pneumococcal MLST database (https://pubmlst.org/spneumoniae/) to obtain the allelic number of each locus and to identify the STs. Clonal complexes were assigned using an eBURST algorithm (http://goeburst.phyloviz.net/).

Phylogeny tree construction

Phylogeny tree was constructed using a neighbor-joining method based on concatenated sequences of seven *S. pneumoniae* housekeeping genes (*aroE, ddl, gdh, gki, recP, spi,* and *xpt*) employing a MEGA 11.0 program (Tamura *et al*, 2021).

Antibiogram profiling

Susceptibility of *S. pneumoniae* serotype 19A isolates to ceftriaxone, meropenem and penicillin was evaluated by a broth microdilution method using cation-adjusted Mueller Hinton broth (Oxoid, Hampshire, United Kingdom) containing 5% lysed horse blood (National Laboratory Animal Center, Mahidol University, Nakhon Pathom Province, Thailand) according to established protocol (CLSI, 2019). Susceptibility to erythromycin (15 µg), levofloxacin (5 µg), ofloxacin (5 µg), sulfamethoxazole/trimethoprim (1.25 µg/23.75 µg), and vancomycin (30 µg) (Oxoid, Hampshire, United Kingdom and Becton Dickinson, Franklin Lakes, NJ) was evaluated using a disk diffusion method according to established protocol (CLSI, 2019). *S. pneumoniae* ATCC 49619 was used as control.

Table 1

Primers used for *Streptococcus pneumoniae* multilocus sequence typing.

Gene	Protein	Amplicon size (bp)	Trimmed size[a] (bp)	Primer sequence (5′→3′)[b]
aroE	Shikimate dehydrogenase	479	405	F: GCCTTTGAGGCGACAGC R: TGCAGTTCA(A/G)AAACAT(A/T)TTCTAA
gdh	Glucose-6-phosphate dehydrogenase	659	459	F: ATGGACAAACCAGC(A/C/G/T)AG(C/T)TT R: GCTTGAGGTCCCAT(A/G)CT(A/C/G/T)CC
gki	Glucose kinase	626	483	F: GGCATTGGAATGGGATCACC R: TCTCCCGCAGCTGACAC
recP	Transketolase	571	448	F: GCCAACTCAGGTCATCCAGG R: TGCAACCGTAGCATTGTAAC
spi	Signal peptidase I	560	472	F: TTATTCCTCCTGATTCTGTC R: GTGATTGGCCAGAAGCGGAA
xpt	Xanthine phosphoribosyl transferase	572	486	F: TTATTAGAAGAGCGCATCCT R: AGATCTGCCTCCTTAAATAC
ddl	D-alanine-D-alanine ligase	513	441	F: TGC(C/T)CAAGTTCCTTATGTGG R: CAC TGG GT(G/A) AAA CC(A/T)GGCAT

[a]Molecular Evolutionary Genetics Analysis version 11 (MEGA 11) software was used to align and trim sequences (Tamura *et al,* 2021)

[b]Enright and Spratt (1998)

bp: base pairs; F: Forward primer; R: Reverse primer

Data analysis

Discrete variables are expressed as percentage, mean ± standard deviation (SD) and proportion. Data were analyzed using a Statistical Package for the Social Sciences (SPSS), version 20 (SPSS Inc, Chicago, IL).

RESULTS

Among a collection of invasive *S. pneumoniae* isolates from 21 hospitals in Thailand during 2008-2018, 62 isolates were identified as serotype 19A by serotype-specific PCR (Fig 1), distributed among 20 distinct MLSTs (Fig 2), seven of which were not previously described in pneumococcal MLST database (http://spneumoniae.mlst.net), the most predominant MLST being ST320, followed by ST2930, ST230, ST63, ST95 and ST8346. These isolates belonged to three clonal complexes, namely, CC63 (36%), CC230 (21%)

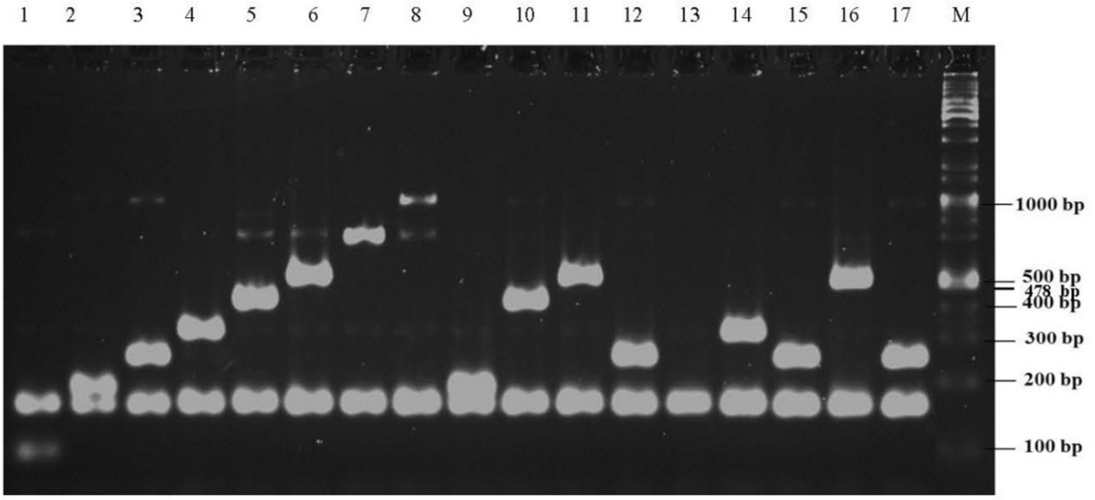

Fig 1 - Identification of clinical *Streptococcus pneumoniae* serotype 19A isolates collected from 21 hospitals in Thailand (2008-2018)

DNA from *S. pneumoniae* isolates were amplified using primers targeting *S. pneumoniae cps*19aK generating a 478-bp amplicon, analyzed by 1% agarose gel-electrophoresis and ethidium bromide staining.

Lane 1: *S. pneumoniae* serotype 4; Lane 2: *S. pneumoniae* serotype 14; Lane 3: *S. pneumoniae* serotype 6; Lane 4: *S. pneumoniae* serotype 19F; Lane 5: *S. pneumoniae* serotype 23F; Lane 6: *S. pneumoniae* serotype 19A; Lane 7: *S. pneumoniae* serotype 12F; Lane 8: *S. pneumoniae* serotype 9V/A; Lanes 9-17: *S. pneumoniae* clinical isolates nos. 20, 21, 23, 25, 27, 26, 28, 29, 30; Lane M: 100-bp DNA size markers (Thermo Fisher Scientific, Waltham, MA)

and CC320 (43%) (Table 2). Phylogeny tree construction demonstrated *S. pneumoniae* serotype 19A isolates were clustered into two groups, Group 1 (containing 22 isolates) and Group 2 (containing 40 isolates) (Fig 3).

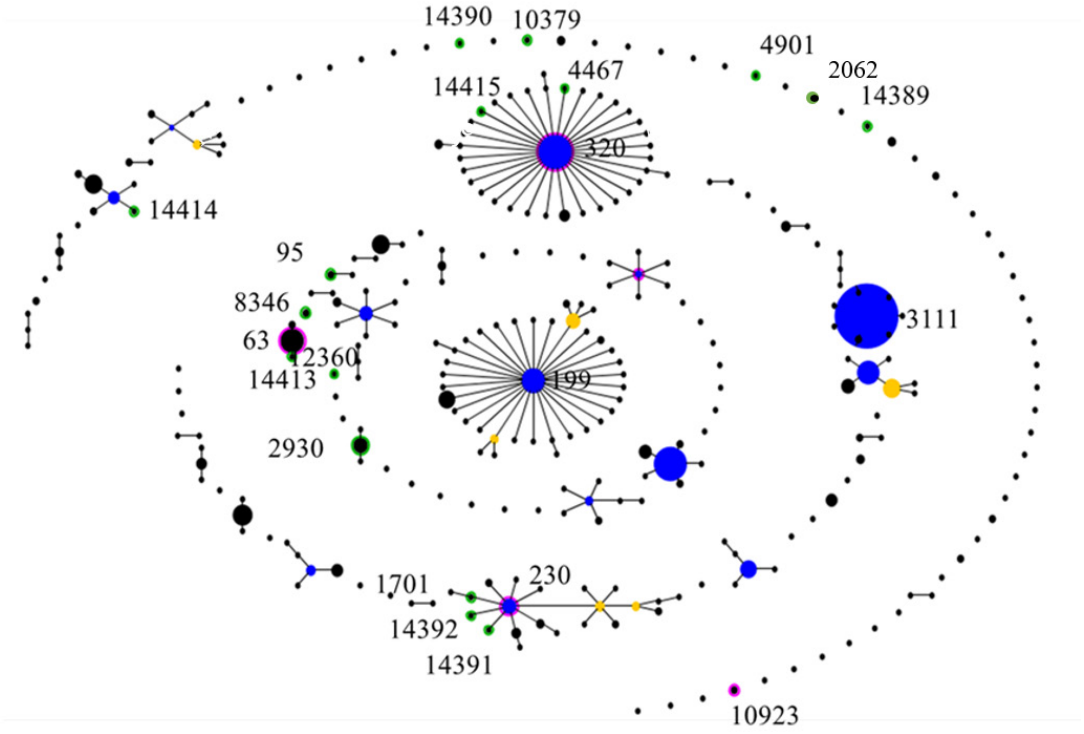

Fig 2 - eBURST diagram of clinical *Streptococcus pneumoniae* serotype 19A isolates collected from 21 hospitals in Thailand (2008-2018)

Sequences of amplicons from seven housekeeping genes (*aroE, ddl, gdh, gki, recP, spi,* and *xpt*) of each *S. pneumoniae* serotype 19A isolate were submitted to the pneumococcal multilocus sequence typing (MLST) database (https://pubmlst.org/spneumoniae/) to obtain allelic number of each locus and to identify MLSTs. Clonal complexes were assigned using an eBURST algorithm (http://goeburst.phyloviz.net/).

Black dot: existing sequence type; pink dot: sequence type found in MLST database; green dot: sequence type in this study; blue dot: sequence type founder; yellow dot: sequence type subgroup founder.

Size of dot corresponds to number of members. Line links single-locus variants. Numeral indicates sequence type.

Table 2

Multilocus sequence types (MLSTs) of clinical *Streptococcus pneumoniae* serotype 19A isolates from 21 hospitals in Thailand (2008-2018)

Clonal complex[a]	MLST[b] (locus allele number[c])	Number of isolates (%) (N = 62)
320	320 (4-16-19-15-6-20-1)	25 (40)
	4467 (4-16-19-15-6-20-14)	1 (1.5)
	14415*(4-16-19-15-6-872-1)	1 (1.5)
230	230 (12-19-2-17-6-22-14)	7 (12)
	14391* (12-19-2-16-6-22-14)	1 (1.5)
	14392* (12-19-2-17-152-22-14)	1 (1.5)
	1701 (12-19-2-1-6-22-14)	3 (6)
63	63 (2-5-36-12-17-21-14)	1 (1.5)
	12360 (4-5-36-12-17-21-14)	9 (13)
	2930 (8-13-8-6-25-6-14)	2 (4)
	95 (5-6-44-2-6-3-4)	2 (4)
	8346 (2-1-36-12-17-31-14)	1 (1.5)
	2062 (1-5-53-32-14-20-199)	1 (1.5)
	4901 (2-8-4-1-6-16-31)	1 (1.5)
	10379 (5-5-87-1-6-1-14)	1 (1.5)
	10923 (185-5-6-10-9-12-75)	1 (1.5)
	14389* (2-508-9-47-6-21-17)	1 (1.5)
	14390* (15-16-19-15-548-20-6)	1 (1.5)
	14413* (15-17-4-16-25-1-17)	1 (1.5)
	14414* (4-4-2-4-4-871-1)	1 (1.5)

*Newly established MLST from the study. The new MLST numbers were assigned by MLST server (https://pubmlst.org/spneumoniae/) curator.

[a]http://goeburst.phyloviz.net; [b]From Fig 2; [c]*aroE-gdh-gki-recP-spi-xpt-ddl* (https://pubmlst.org/spneumoniae/)

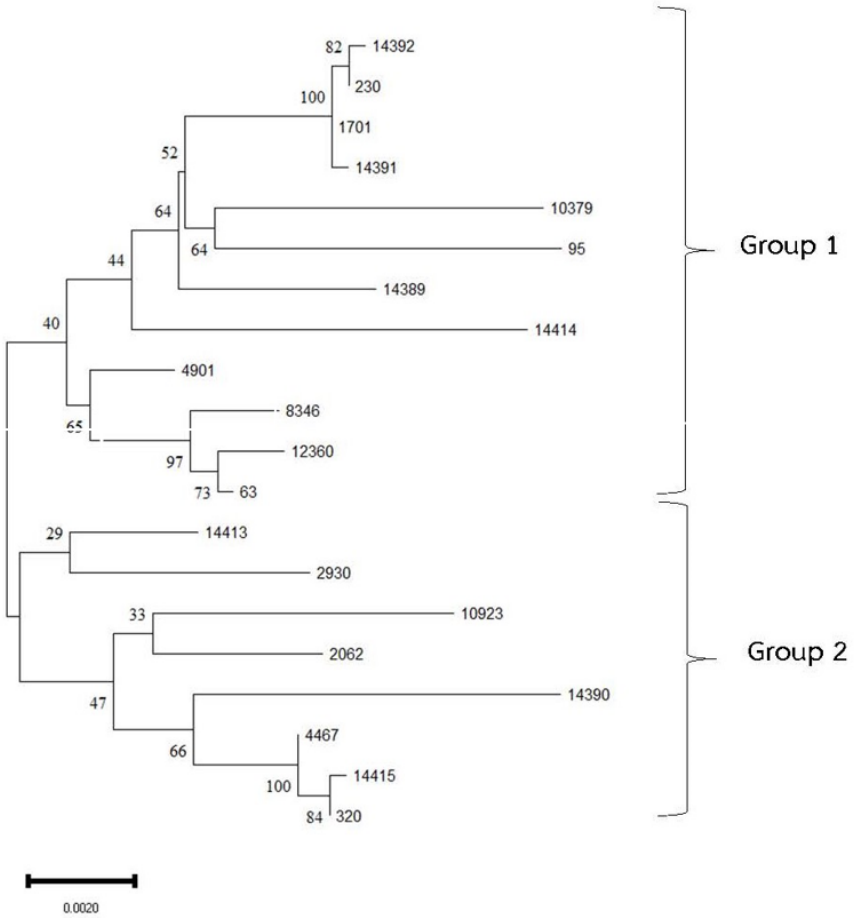

Fig 3 - Phylogeny tree of clinical *Streptococcus pneumoniae* serotype 19A isolates collected from 21 hospitals in Thailand (2008-2018)

Phylogeny tree was constructed using a neighbor-joining method based on concatenated sequences of seven *Streptococcus pneumoniae* housekeeping genes (*aroE, ddl, gdh, gki, recP, spi,* and *xpt*) employing the MEGA 11.0 program (Tamura et al, 2021). A bootstrap analysis was conducted using 1,000 replicates. Groups 1 and 2 contained 22 and 40 isolates respectively. Numeral indicates sequence type and number at node percent of bootstrap value (indicates how many times out of 1,000 the same branch was observed when repeating the phylogenetic reconstruction). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogeny tree construction demonstrated *S. pneumoniae* serotype 19A isolates were clustered into two groups by standing on the different branches of the major topology; Group 1 includes ST14392, 230, 1701, 14391, 10379, 95, 14389, 14414, 4901, 8346, 12360, and 63 (support by bootstrap value = 40%) while Group 2 includes ST14413, 2930, 10923, 2062, 14390, 4467, 14415, and 320 (no bootstrap support).

Antimicrobial susceptibility testing by broth microdilution and disk diffusion assay showed only isolate (ID51) (1.5%) was susceptible to all seven drugs or drug combinations tested, 11 (18%) isolates were susceptible to six drugs and 14 (22%) isolates to five drugs (Table 3). All isolates tested were susceptible to levofloxacin, ofloxacin and vancomycin. MDR phenotypes were found in 33 (53.2%) isolates. Among isolates from patients with meningitis, 93 and 52% were resistant or intermediate resistant to penicillin and cefotaxime/ceftriaxone respectively.

DISCUSSION

Prevalence of IPD caused by penicillin-resistant *S. pneumoniae* serotype 19A has increased worldwide (Cassiolato *et al*, 2018; Rockett *et al*, 2018; Corcoran *et al*, 2021). There are multiple theories associated with an increase of serotype 19A, such as induction by PCV7 of serotype replacement or serotype switching, antibiotic pressure, and introduction of new clones (Reinert *et al*, 2010). In areas of South Korea where PCV7 was not available or was sporadically used an increase of serotype 19A was observed even before the vaccine was widely introduced (Choi *et al*, 2008). In Thailand, PCV7 was introduced in 2006 and prescribed upon physician's recommendation or family request (Srifuengfung *et al*, 2010). Prevalence of serotype 19A among children ≤5 years of age is 5.6% during the period 2000-2009, increasing to 18.3% during 2009-2012, and continues to increase up to 25.0% in 2012 in spite of low use of PCVs, becoming the most common serotype in patients 6-64 years of age during 2012-2016 (Phongsamart *et al*, 2014), supporting the notion that other factors than PCVs are involved in this rise in prevalence (Gagetti *et al*, 2017). Importantly, 19A serotype is less susceptible to antimicrobial agents compared to non-19A isolates (Hawkins *et al*, 2017; Ruiz Garcia *et al*, 2021).

eBURST analysis of MLST database demonstrates a relationship among *S. pneumoniae* STs and CCs, with a CC being composed of one major serotype, but a CC may contain several serotypes, indicating occurrence of serotype switching (Reinert *et al*, 2010). Five major CCs exist among serotype19A isolates including ST81, ST193, ST199, ST276 and ST320 (Reinert *et al*, 2010). Our study demonstrated that from 2008 to 2018 the increase in number of *S. pneumoniae* serotype 19A was the result of a clonal expansion of ST320/CC320 associated with MDR phenotype. This finding is in concordance with a USA study in 2005 reporting the most common penicillin-resistant serotype19A following PCV7 introduction belongs to an emerging CC320, followed by the pre-existing CC199 (Moore *et al*, 2008); three years later, prevalence of CC320/271 has increased while CC199 decreased (Beall

Table 3

Antibiogram profile, clonal complex (CC) and multilocus sequence type (MLST) of clinical *Streptococcus pneumoniae* serotype 19A isolates collected from 21 hospitals in Thailand (2008-2018)

Isolate ID	Antibiotic susceptibility												CC	MLST	Hospital	Year of isolation
	Broth microdilution method[a]								Disk diffusion assay[b]							
	Penicillin			CTX/CRO			MEM		EM	SXT	OFX/LFX	VA				
	MIC (μg/ml)	Men⁻ S/I/R	Men⁺ S/I/R	MIC (μg/ml)	Men⁻ S/I/R	Men⁺ S/I/R	MIC (μg/ml)	S/I/R	S/I/R	S/I/R	S/I/R	S/I/R				
1	0.03	S	S	0.50	S	S	1.00	R	R	S	S	S	63	63	Bhumibol	2009
2	0.008	S	S	0.006	S	S	0.06	S	R	S	S	S	63	63	Siriraj	2012
3	4.00	I	R	1.00	S	I	0.50	I	R	S	S	S	63	63	NA	2014
4	4.00	I	R	1.00	S	I	2.00	R	R	R	S	S	Singleton	95	Siriraj	2011
5	0.25	S	R	0.25	S	S	0.125	S	R	R	S	S	Singleton	95	NA	2013
6	0.19	S	R	0.06	S	S	1.00	R	S	R	S	S	230	230	Siriraj	2012
7	0.125	S	R	0.125	S	S	0.50	I	S	R	S	S	230	230	Siriraj	2012
8	0.25	S	R	0.25	S	S	0.50	I	S	R	S	S	230	230	Siriraj	2012
9	0.50	S	R	0.125	S	S	0.50	I	S	R	S	S	230	230	NA	2014
10	0.38	S	R	0.125	S	S	0.06	S	S	R	S	S	230	230	NA	2015
11	0.125	S	R	0.06	S	S	0.06	S	S	R	S	S	230	230	NA	2015
12	-	-	-	-	-	-	-	-	S	R	-	S	230	230	Siriraj	2017
13	4.00	I	R	1.00	S	I	4.00	R	R	R	S	S	320	320	Siriraj	2008
14	2.00	S	R	1.00	S	I	4.00	R	R	R	S	S	320	320	Siriraj	2010
15	1.50	S	R	1.50	I	R	4.00	R	R	R	S	S	320	320	Siriraj	2011

Table 3 (cont)

Isolate ID	Antibiotic susceptibility												CC	MLST	Hospital	Year of isolation
	Broth microdilution method[a]								Disk diffusion assay[b]							
	Penicillin			CTX/CRO			MEM		EM	SXT	OFX/LFX	VA				
	MIC (μg/ml)	Men$^-$ S/I/R	Men$^+$ S/I/R	MIC (μg/ml)	Men$^-$ S/I/R	Men$^+$ S/I/R	MIC (μg/ml)	S/I/R	S/I/R	S/I/R	S/I/R	S/I/R				
16	0.016	S	S	0.016	S	S	4.00	R	S	S	S	S	320	320	Nopparat	2011
17	2.00	S	R	1.50	I	R	4.00	R	R	R	S	S	320	320	Nopparat	2012
18	1.00	S	R	1.00	S	I	2.00	R	I	R	S	S	320	320	Charoenkrung	2012
19	4.00	I	R	1.00	S	I	2.00	R	R	R	S	S	320	320	Vejtani	2012
20	4.00	I	R	1.00	S	I	0.25	S	S	R	S	S	320	320	Nopparat	2012
21	4.00	I	R	0.25	S	S	1.00	R	R	R	S	S	320	320	Siriraj	2012
22	4.00	I	R	2.00	I	R	1.00	R	R	R	S	S	320	320	NA	2013
23	0.50	S	R	0.125	S	S	0.06	S	S	R	S	S	320	320	NA	2013
24	1.00	S	R	0.50	S	S	0.25	S	R	R	S	S	320	320	NA	2013
25	0.75	S	R	0.75	S	I	0.25	S	R	R	S	S	320	320	NA	2013
26	1.00	S	R	1.00	S	I	0.50	I	R	R	S	S	320	320	NA	2014
27	8.00	R	R	8.00	R	R	1.00	R	R	R	S	S	320	320	NA	2014
28	0.75	S	R	0.75	S	I	0.50	I	R	R	S	S	320	320	NA	2014
29	1.00	S	R	1.50	I	R	0.50	I	R	R	S	S	320	320	NA	2015
30	1.00	S	R	0.25	S	S	0.25	S	R	R	S	S	320	320	NA	2015
31	2.00	S	R	1.00	S	I	0.50	I	R	R	S	S	320	320	NA	2015
32	4.00	I	R	1.00	S	I	0.50	I	R	R	S	S	320	320	NA	2015

Table 3 (cont)

Isolate ID	Antibiotic susceptibility												CC	MLST	Hospital	Year of isolation
	Broth microdilution method[a]								Disk diffusion assay[b]							
	Penicillin			CTX/CRO			MEM		EM	SXT	OFX/LFX	VA				
	MIC (μg/ml)	Men^- S/I/R	Men^+ S/I/R	MIC (μg/ml)	Men^- S/I/R	Men^+ S/I/R	MIC (μg/ml)	S/I/R	S/I/R	S/I/R	S/I/R	S/I/R				
33	4.00	I	R	1.00	S	I	2.00	R	R	R	S	S	320	320	Siriraj	2017
34	4.00	I	R	1.00	S	I	2.00	R	R	R	S	S	320	320	Siriraj	2017
35	2.00	S	R	0.50	S	S	2.00	R	S	S	S	S	320	320	Charoenkrung	2017
36	4.00	I	R	1.00	S	I	2.00	R	R	R	S	S	320	320	Siriraj	2018
37	4.00	I	R	1.00	S	I	2.00	R	R	R	S	S	320	320	Siriraj	2018
38	0.25	S	R	0.094	S	S	0.50	I	S	R	S	S	230	1701	Siriraj	2012
39	-	-	-	-	-	-	-	-	S	R	-	S	Singleton	2062	Siriraj	2017
40	3.00	I	R	0.75	S	I	2	R	R	R	S	S	Singleton	2930	Nopparat	2012
41	1.50	S	R	1.00	S	I	0.50	I	R	R	S	S	Singleton	2930	NA	2014
42	4.00	I	R	0.25	S	S	0.50	I	R	R	S	S	Singleton	2930	NA	2015
43	2.00	S	R	1.00	S	I	1.00	R	R	R	S	S	Singleton	2930	Bhumibol	2017
44	2.00	S	R	1.00	S	I	1.00	R	R	R	S	S	Singleton	2930	Bhumibol	2017
45	2.00	S	R	0.03	S	S	1.00	R	R	R	S	S	Singleton	2930	Bhumibol	2017
46	4.00	I	R	1.00	S	I	2.00	R	R	R	S	S	Singleton	2930	Bhumibol	2017
47	4.00	I	R	1.00	S	I	1.00	R	R	R	S	S	Singleton	2930	Bhumibol	2018
48	2.00	S	R	0.50	S	S	1.00	R	R	R	S	S	Singleton	2930	Bhumibol	2018
49	0.125	S	R	0.06	S	S	0.06	S	S	R	S	S	320	4467	NA	2016

Table 3 (cont)

Isolate ID	Antibiotic susceptibility												CC	MLST	Hospital	Year of isolation
	Broth microdilution method[a]								Disk diffusion assay[b]							
	Penicillin			CTX/CRO			MEM		EM	SXT	OFX/LFX	VA				
	MIC (μg/ml)	Men$^-$ S/I/R	Men$^+$ S/I/R	MIC (μg/ml)	Men$^-$ S/I/R	Men$^+$ S/I/R	MIC (μg/ml)	S/I/R	S/I/R	S/I/R	S/I/R	S/I/R				
50	2.00	S	R	0.50	S	S	0.50	I	S	S	S	S	Singleton	4901	NA	2014
51	0.125	S	R	0.125	S	S	0.06	S	S	S	S	S	Singleton	8346	NA	2014
52	0.094	S	R	0.06	S	S	0.03	S	R	S	S	S	Singleton	8436	NA	2014
53	0.75	S	R	0.38	S	S	0.03	S	R	R	S	S	Singleton	10379	Nopparat	2012
54	4.00	I	R	1.00	S	I	0.25	S	S	I	S	S	Singleton	10923	Bhumibol	2009
55	2.00	S	R	0.25	S	S	0.50	I	R	S	S	S	63	12360	NA	2014
56	1.50	S	R	1.00	S	I	4.00	R	R	R	S	S	Singleton	14389	Rama 9	2011
57	0.50	S	R	1.00	S	I	0.50	I	R	R	S	S	Singleton	14390	Thainakarin	2017
58	0.047	S	S	0.047	S	S	0.03	S	S	R	S	S	230	14391	Siriraj	2012
59	0.50	S	R	0.12	S	S	0.50	I	R	R	S	S	230	14392	NA	2013
60	0.50	S	R	0.38	S	S	4.00	R	R	I	S	S	Singleton	14413	Siriraj	2011
61	2.00	S	R	0.25	S	S	0.25	S	S	R	S	S	Singleton	14414	NA	2014
62	1.00	S	R	0.75	S	I	0.06	S	R	R	S	S	320	14415	NA	2016

[a]Cation-adjusted Mueller Hinton broth (Oxoid, Hampshire, United Kingdom) containing 5% lysed horse blood (National Laboratory Animal Center, Mahidol University, Nakhon Pathom Province, Thailand) according to CLSI (2019); [b]According to CLSI (2019)

CTX/CRO: cefotaxime/ceftriaxone; EM: erythromycin (15 μg); I: intermediate resistant; MEM: meropenem; Men$^+$: with meningitis; Men$^-$: without meningitis; MIC: minimum inhibitory concentration; NA: not available; OFX/LFX: ofloxacin/levofloxacin (5 μg/5μg); R: resistant; S: susceptible; SXT: sulfamethoxazole/ trimethoprim (1.25 μg/23.75 μg); VA: vancomycin (30 μg); - means unable to determine due to death of organisms

et al, 2011). Similarly, in 11 Asian countries MDR ST320 is associated with the increase in serotype 19A prevalence (Choi *et al*, 2008; Shin *et al*, 2011). A study in competitive pneumococcal colonization of nasopharynx in a murine model showed ST320 clone (a double locus variant of ST236) derived from an ancestral Taiwan 19F-14 (ST236), revealed that serotype 19F ST320 has a lower capacity than serotype 19A ST320 (Hsieh *et al*, 2013; Corcoran *et al*, 2021). Thus, evolution of serotype 19A ST320 clone not only changes capsular serotype but also alters other genetic loci (Hsieh *et al*, 2013). In Alaska USA, Italy and Norway the most predominate clonal cluster is CC199, which is already present before PCV7 implementation (Vestrheim *et al*, 2012; Camilli *et al*, 2013; Rudolph *et al*, 2013). However, CC199 was not detected in our study and we could not compare changes in 19A serotype genetic structure before and after PCV7 introduction due to a lack in data of isolates before PCV7 introduction among the study samples.

The second most common ST in the study, ST2930, according to eBURST analysis was a single locus variant (SLV) of ST2218 (in turn a SLV of ST176). ST2930 was first isolated in 1999 from a patient from Israel with acute otitis media and demonstrated a penicillin MIC of 1 μg/ml (Dagan *et al*, 2009). There is no subsequent report of ST2930 until this work; all isolates were MDR.

The third most ST in this study, ST230/CC230, was a clone of Denmark14-ST230, a cause of pneumococcal infection in Portugal (Aguiar *et al*, 2010) and the only common clone among MDR 19A isolates in Germany prior to PCV7 (van der Linden *et al*, 2013). Following introduction of PCV7, in France there is an increase in the spread of ST276, a SLV of ST230, with intermediate resistance to penicillin (Mahjoub-Messai *et al*, 2009), and in Spain ST276 became the third most common penicillin-resistant serotype 19A (Ardanuy *et al*, 2009). Our study observed 14.3% of serotype19A ST276 isolates were non-susceptible to penicillin.

The most predominant ST among serotype19A isolates from Maela refugee camp, Tak Province, Thailand (https://pubmlst.org/spneumoniae/) causing either community-acquired or carriage of pneumonia is ST1701, followed by ST230, ST5227 and ST5661, supporting the supposition that ST230 and ST1701 found in their study was due to their high prevalence among asymptomatic subjects. CC3111 is the most predominant clonal cluster in Japan (https://pubmlst.org/spneumoniae/) but was not found in our study. On the other hand, ST320 was not previously reported in Thailand prior to the current study. In addition, molecular characteristics of serotype19A isolates presented here was different from the 2011 ANSORP study (Shin *et al*, 2011), *ie*, the most

common clonal complex in their study was CC172 and there were 6 MLSTs while in this study, the most common clonal complex was CC320 and there were 20 MLSTs.

In conclusion, the study demonstrates the highly antimicrobial-resistant *Streptococcus pneumoniae* serotype 19A ST320 and ST2930 isolates were disseminated among the 21 hospitals surveyed in 2008-2018, with the *caveat* that the data are valid only for the regions and period of collection. Continued surveillance of *S. pneumoniae* 19A remains an important aspect in the control and prevention of this invasive pneumococcal disease in Thailand.

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CONFLICTS OF INTEREST DISCLOSURE

The authors declare no conflicts of interest.

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