CHARACTERIZATION OF AMPICILLIN-RESISTANCE IN *HAEMOPHILUS INFLUENZAE* AND SUSCEPTIBILITY TO KAFFIR LIME OIL

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ABSTRACT

Haemophilus influenzae is one of the most common bacteria causing respiratory tract infections. Beta-lactams and macrolides are the two most common antibiotics in the treatment of such infection. Beta-lactams are drugs of choice for *H. influenzae*. Although it is usually susceptible to these drugs, resistance mechanisms lead to important problems.

In this study, ampicillin-resistance in *H. influenzae* was characterized by phenotypic and genotypic methods. The bla_{TEM} (99%) and bla_{ROB-1} (1%) genes found in *H. influenzae* produced beta-lactamase. The minimal inhibitory concentration (MIC) of ampicillin ranged from 8 - > 512 µg/ml. Another resistance mechanism is by mutation in the *ftsI* gene encoded for altered penicillin binding protein 3 (PBP3) that has low affinity for beta-lactams identified in beta-lactamase negative, ampicillin-resistant isolates (BLNAR). Twenty-six BLNAR were isolated and MIC of ampicillin and amoxicillin/clavulanic acid for BLNAR ranged from 2 - 16 µg/ml and 0.75 - 4 µg/ml, respectively. The prevalence of BLNAR isolates from *H. influenzae* infected patients in Siriraj Hospital increased from 3.95% in 2008 to 8.39% in 2010. From drug susceptibility testing, beta-lactamase producing isolates were susceptible to third generation cephalosporins, e.g., cefotaxime and ceftriaxone, whereas BLNAR isolates were less susceptible to these drugs. DNA sequencing in an isolate of bla_{TEM} showed 100% nucleotide and amino acid sequence identity with the published sequence of the bla_{TEM-1} gene. DNA sequencing in 10 BLNAR showed mutations in various positions of PBP3. These mutations were classified in group II according to Ubukata, et al. Six patterns of amino acid substitutions were found, of which mutations in M314V and A561E were new findings in this study.

Antibacterial activity of kaffir lime leaf oil and kaffir lime oil against respiratory tract pathogens were studied. The results showed antibacterial activity of kaffir lime leaf oil and kaffir lime oil against *H. influenzae*, Group A, B, C, F, G streptococci, *S. pneumoniae*, *S. aureus* (MRSA and MSSA) and *A. baumannii* by disk diffusion and broth microdilution methods. MIC ranged from 0.06 - 68 and 0.06 - 17.4 mg/ml for kaffir lime leaf oil and kaffir lime oil, respectively. The results showed that citronellal, the major component in kaffir lime leaf oil, was an active ingredient while limonene, one of the components in kaffir lime oil, may have synergistic effects with other components causing antibacterial effects. These tested oils had stability up to 4 months.

This study provided data about ampicillin-resistance characterization in *H. influenzae*, which is necessary to monitor the prevalence of BLNAR for proper use of antibacterial agents, and antibacterial activity from oils of kaffir lime which may lead to the usage of this traditional herb as an antibacterial agent, and product development in order to provide an alternative choice for treatment and to control drug resistant bacteria.

KEY WORDS: *HAEMOPHILUS INFLUENZAE*/ BETA-LACTAMASE/ PENICILLIN BINDING PROTEIN 3/ KAFFIR LIME LEAF OIL/ KAFFIR LIME OIL

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ลักษณะของการดื้อยาแอมพิซิลลินในเชื้อ *Haemophilus influenzae* และความไวของเชื้อนี้ต่อน้ำมันมะกรูด

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บทคัดย่อ

Haemophilus influenzae เป็นเชื้อก่อโรคที่สำคัญในระบบทางเดินหายใจ ยาในกลุ่มเบต้า-แลคแตมและแมคโครไลด์ คือ ยาที่ใช้ในการรักษาการติดเชื้อนี้ โดยยาในกลุ่มเบต้า-แลคแตม เป็นยาที่เหมาะสมที่สุดในการรักษาโรคติดเชื้อ *Haemophilus influenzae* แม้ว่าเชื้อดังกล่าวจะไวต่อยาชนิดนี้ แต่การดื้อยาเบต้า-แลคแตมก็เป็นปัญหาที่สำคัญ

การศึกษาครั้งนี้ได้ทำการทดสอบลักษณะการดื้อยาแอมพิซิลลิน ด้วยวิธีทางกายภาพและทางพันธุกรรม โดยพบว่ามียีน bla_{TEM} 99% และ bla_{ROB-1} 1% ในเชื้อ *Haemophilus influenzae* สายพันธุ์ที่สร้างเอนไซม์เบต้า-แลคตาเมส ซึ่งพบว่ามีค่าความเข้มข้นที่น้อยที่สุดที่สามารถยับยั้งการเจริญของเชื้อ (MIC) ต่อยาแอมพิซิลลิน คือ 8 - > 512 µg/ml ส่วนการดื้อยากลุ่มเบต้า-แลคแตมด้วยกลไกอื่น คือ การเกิดการกลายพันธุ์ในยีน *ftsI* ซึ่งทำให้ความสามารถในการจับกับยาลดลง คือ เชื้อสายพันธุ์ที่ไม่สร้างเอนไซม์เบต้า-แลคตาเมสและดื้อยาแอมพิซิลลิน (BLNAR) ซึ่งมีจำนวน 26 สายพันธุ์ และพบว่า MIC ของยาแอมพิซิลลิน และยาอะม็อกซีซิลลิน คลาวูลานิก แอซิดมีค่าตั้งแต่ 2 - 16 และ 0.75 - 4 µg/ml ตามลำดับ การศึกษายังพบอีกว่า ความชุกของสายพันธุ์ BLNAR ในผู้ป่วยที่ติดเชื้อ *Haemophilus influenzae* ในโรงพยาบาลศิริราช เพิ่มขึ้นจาก 3.95% ในปี ค.ศ. 2008 เป็น 8.39% ในปี ค.ศ. 2010 สำหรับการทดสอบความไวต่อยา พบว่าสายพันธุ์ที่สร้างเอนไซม์เบต้า-แลคตาเมสมีความไวต่อยาในกลุ่มเซฟาโลสปอริน รุ่นที่ 3 เช่น เซโฟแทกซี และ เซฟไตรอะโซน ขณะที่ BLNAR มีความไวต่อยาที่ใช้ทดสอบเหล่านี้น้อยกว่าสายพันธุ์ที่สร้างเอนไซม์เบต้า-แลคตาเมส สำหรับการหาชนิดของเอนไซม์ TEM พบว่าเป็นชนิด TEM-1 ขณะที่การกลายพันธุ์ที่เกิดขึ้นใน BLNAR 10 สายพันธุ์ พบว่าเกิดขึ้นในหลายๆ ตำแหน่งของ PBP3 ซึ่งการกลายพันธุ์ทั้งหมดที่เกิดขึ้นอยู่ในกลุ่ม 2 ตามการแบ่งประเภทโดย Ubukata และคณะ นอกจากนี้ยังพบ การเปลี่ยนของกรดอะมิโน 6 แบบและพบการกลายพันธุ์ที่ M314V และ A561E เป็นครั้งแรก

การทดสอบการยับยั้งเชื้อแบคทีเรียที่ก่อโรคในระบบทางเดินหายใจด้วยน้ำมันจากใบและผลมะกรูด พบว่าน้ำมันจากใบและผลมะกรูดมีผลยับยั้งเชื้อ *Haemophilus influenzae*, Group A, B, C, F, G streptococci, *S. pneumoniae*, *S. aureus* (MRSA and MSSA) and *A. baumannii* ด้วย MIC 0.06 - 68 และ 0.06 - 17.4 mg/ml สำหรับน้ำมันใบและผลมะกรูดตามลำดับ นอกจากนี้พบว่า citronellal ในน้ำมันใบมะกรูดเป็นสารที่ทำหน้าที่ออกฤทธิ์ ส่วน limonene ในน้ำมันผลมะกรูดน่าจะเกิดการเสริมฤทธิ์กับสารประกอบอื่นๆ และพบว่าน้ำมันจากมะกรูดมีความคงตัวจนถึง 4 เดือนที่ทำการทดสอบ

การศึกษานี้ได้แสดงข้อมูลเกี่ยวกับลักษณะการดื้อยาแอมพิซิลลินในเชื้อ *Haemophilus influenzae* ซึ่งจำเป็นในการติดตามความชุกของ BLNAR และการเลือกใช้ยาที่เหมาะสม ส่วนการทดสอบการยับยั้งเชื้อโดยน้ำมันจากมะกรูดอาจจะนำไปสู่การพัฒนาผลิตภัณฑ์เพื่อเป็นอีกหนึ่งทางเลือกสำหรับการรักษาและควบคุมเชื้อดื้อยา

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LIST OF ABBREVIATIONS

ABBREVIATION	TERM
ATCC	American Type Culture Collection
bla	Structural gene of beta-lactamase
BLAST	basic local alignment search tool
BLPAR	beta-lactamase positive, ampicillin-resistant
BLNAR	beta-lactamase negative, ampicillin-resistant
BLPACR	beta-lactamase positive, amoxicillin/clavulanic acid-resistant
bp	base pair
dNTP	deoxyribonucleotide triphosphate
kb	kilobase pair (10^3 base pair)
kDa	kilodalton (10^3 Dalton)
M	molar
mM	millimolar (10^{-3} molar)
RFLP	restriction fragment length polymorphism
ROB	an Ambler class A enzyme first isolated from a patient 'Rubin'
TEM	an Ambler class A enzyme first isolated from a patient 'Temoniera'
UV	ultraviolet
Ala, A	Alanine
Arg, R	Arginine
Asn, N	Asparagine
Asp, D	Aspartate

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LIST OF ABBREVIATIONS (cont.)

ABBREVIATION	TERM
Cys, C	Cysteine
Gln, Q	Glutamine
Glu, E	Glutamate
Gly, G	Glycine
His, H	Histidine
Ile, I	Isoleucine
Leu, L	Leucine
Lys, K	Lysine
Met, M	Methionine
Phe, F	Phenylalanine
Pro, P	Proline
Ser, S	Serine
Thr, T	Threonine
Trp, W	Tryptophan
Tyr, Y	Tyrosine
Val, V	Valine

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CHAPTER I
INTRODUCTION

Haemophilus influenzae, a major respiratory tract pathogen found in respiratory tract as commensals and invasive pathogens. There are six encapsulated types, a through f, as well as noncapsulated or untypable isolates (1). It causes disease e.g., pneumonia, acute otitis media and meningitis (2), in which the childhood pneumonia, meningitis, and bacteremia are caused by type b isolates, whereas community-acquired pneumonia in adults, acute otitis media, acute sinusitis, and acute exacerbation of chronic bronchitis are typically caused by untypable isolates (1). *H. influenzae* type b (Hib) can cause meningitis in young children. Prior to introduction of Hib vaccine, Hib was estimated to be responsible worldwide for three million serious illnesses and 386,000 deaths per year, through meningitis and pneumonia (3).

Although the bacterium is usually sensitive to beta-lactams, the major mechanism of beta-lactam resistance is production of beta-lactamase referred as beta-lactamase positive, ampicillin resistant isolates (BLPAR). Another mechanism is the presence of altered penicillin binding proteins (PBPs) with lowered affinity for beta-lactams referred as beta-lactamase negative, ampicillin resistant isolates (BLNAR) and a very small proportion of isolates possess both beta-lactamase production and PBPs alteration mechanisms referred as beta-lactamase positive, amoxicillin/clavulanate resistant isolates (BLPACR) (4-7). In *H. influenzae*, susceptibility to all beta-lactams is generally predicted by susceptibility to ampicillin as defined by CLSI minimal inhibitory concentration (MIC) breakpoints which are as follows: ≤1 μg/ml susceptible, 2 μg/ml intermediate and ≥4 μg/ml resistant (8).

Beta-lactamase mediated ampicillin resistance (BLPAR) isolates in *H. influenzae* was first detected in the early 1970s, and the beta-lactamase was identified as a TEM type in 1975 (9) which most evidence indicates that the TEM beta-lactamase is TEM-1 (10, 11). In 1981, Rubin et al. reported the presence of a novel beta-lactamase in *H. influenzae*, later called ROB-1 (12). Some studies found 93.7% TEM-

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1 and 4.6% ROB-1 in a global survey of over 2,000 beta-lactamase positive isolates (10, 13, 14). TEM and ROB-1 beta-lactamases are plasmid-mediated class A serine beta-lactamases with similar substrate profiles that confer resistance to ampicillin and are effectively inhibited by beta-lactamase inhibitor (such as clavulanate). In some countries, the prevalence of beta-lactamase positive isolates have begun to decline e.g., 32% in 1993 to 19% in 2000 in Canada (6); 28% in 1998 to 16% in 2000 in Spain (7); 36% in 1994 to 26% in 2002 in USA (6, 15); and 25% in 1995 to 3% in 1999 in Japan (16). While the prevalence of altered PBPs with BLNAR isolates remains low globally. However, there are some countries where these isolates are increasing, especially in Japan (16). According to Japanese nationwide surveillance study (16) conducted in 1997 and 1998, the proportion of clinical isolates supposed to be BLNAR has rapidly increased to 28.8% and still increasing every year (17). Moreover, especially in children, the frequency of BLNAR is rapidly increasing e.g., increasing to 8.9% during the last 7-month period in Japan from January 1997 to July 1999 (18).

The first observation of BLNAR isolates was reported in 1977 (17). BLNAR isolate is beta-lactamase negative with an ampicillin MIC above CLSI resistant breakpoint of ≥4 µg/ml. The beta-lactamase negative isolate with an ampicillin MIC of 2 µg/ml is often included in BLNAR category (19). BLNAR isolates acquire drug resistance via mutations in the *ftsI* gene encoding penicillin-binding protein (PBP) 3, which is associated with septal peptidoglycan synthesis, the major component of bacterial cell wall (20). Amino acid substitutions in PBP3 surrounding the conserved Lys512-Thr-Gly (KTG) and Ser379-Ser-Asn (SSN) motifs are responsible for beta-lactam resistance (16, 21-24). BLNAR isolates can be categorized not just on the ampicillin MIC but also by the use of PCR and sequencing to detect specific mutations in the *ftsI* gene. They can be categorized as low BLNAR and high BLNAR. Low-BLNAR isolates have single substitution of Asn526Lys or Arg517His with intermediate resistance to ampicillin usually have ampicillin MICs ranged 0.5-2 µg/ml, whereas high BLNAR isolates have amino acid substitution of Asn526Lys or Arg517His in addition with Met377Ile, Ser385Thr, and/or Leu389Phe with ampicillin MICs ranged 1-16 µg/ml (16, 25, 26).

Thailand is located in geography which there is various kinds of plants. Many medicinal herbs for daily life usage in Thailand have been known as ‘Food are

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Medicine' in the folk medicine (27). Essential oils are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). The antimicrobial properties of essential oils and their constituents from a wide variety of plants have been assessed that these plant secondary metabolites have potential in medical procedures and applications in cosmetic, food and pharmaceutical industries (28). The activity of oils would be expected to relate to respective composition of oils, the structural configuration of the constituent components of oils and possible synergistic interactions between components (28). Many components in essential oils can kill bacteria e.g., estragole, citral, thymol, eugenol, geraniol and isoeugenol.

Kaffir lime or *Citrus hystrix*, is a Thai herb. It is small tree, rough, warty green fruit. The valued parts are the leaves (bai magrood) and the peel (pew magrood) or zest of the limes. In general, kaffir lime leaf oil contains citronellal (70%) whereas kaffir lime peel oil contains beta-pinene (30-40%), limonene (24-30%). Kaffir lime has many therapeutic benefits e.g., the extract helps increasing blood circulation, the fruit and leaves are excellent for promoting skin regeneration, the juice can be used to treat dandruff and hair loss and encourage healthy hair growth. Kaffir lime also acts as an excellent rejuvenation, reducing colds, and helps regaining energy and dispelling tiredness. Moreover, it acts as antibacterial effect such kaffir lime leaf, kaffir lime peel and clove oil exhibited greater antibacterial activity against 20 serotypes of *Salmonella* and 5 species of other enterobacteria, compared to other spice oils (29). The oil extracts of kaffir lime leaf and khaminkhow showed high activities, when chilli padi, homdaeng and ginger showed moderate activities against *S. aureus* and all oil extracts of kaffir lime's leaves, khaminkhow, chilli padi, homdaeng and ginger also showed moderate activities against *S. typhi* (27). Kaffir lime leaf, lemongrass, clove and michelia oil have the strongest antibacterial activity against 5 isolates of *Propionibacterium acnes* (30). Ajowan seed oil exhibited the most antibacterial activity followed by coriander seed, nutmeg, caraway seed, kaffir lime, mace and kaempferia oil against extended-spectrum beta-lactamase producing *Klebsiella pneumoniae* (31).

This study was performed to identify beta-lactamase producing and BLNAR *H. influenzae* isolates and determine antimicrobial susceptibility that was

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performed by nitrocefin method (8) and determination of MIC values for ampicillin in beta-lactamase producing isolates by broth microdilution (8). MIC values for ampicillin and amoxicillin/clavulanate in BLNAR isolates were performed by broth microdilution and E-test methods, respectively. Type of beta-lactamase producing and mutations in *ftsI* gene encoding PBP3 of BLNAR isolates were determined by molecular techniques (PCR and DNA sequencing methods). In addition, determination the inhibitory effect of kaffir lime leaf oil and kaffir lime oil on both susceptible and resistant *H. influenzae* isolates were studied. Moreover, other bacteria causing respiratory tract infection i.e., Group A streptococci, *Streptococcus pneumoniae*, *Staphylococcus aureus* (MRSA and MSSA), *Acinetobacter baumannii* were studied by disk diffusion and broth microdilution methods.

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CHAPTER II
LITERATURE REVIEW

Haemophilus influenzae was first isolated and named in 1890 by Pfeiffer who mistakenly thought it was the cause of influenza pandemic (32). It contains six capsulated types which are type a through type f and noncapsulated or untypeable isolates (1). It is one of the major bacteria of respiratory tract infections in children and adults (1). Major diseases caused by *H. influenzae* are childhood pneumonia, meningitis, bacteremia, community-acquired pneumonia, acute otitis media, acute sinusitis and acute exacerbations of chronic bronchitis (1). It is the first organism where vaccine made of its polysaccharide capsule conjugated to carrier protein successfully activated protective immunity in children (33). *H. influenzae* resistance to beta-lactam antibiotics is an increasing problem. The resistance to ampicillin in this organism varies from 10% to 60%, depending on geographical region (34). *H. influenzae* can acquire ampicillin resistance through two different mechanisms. One is the production of beta-lactamases of TEM-1 type (35) and ROB-1 type (36). Another mechanism is a conformational change in penicillin-binding proteins (PBPs), which are enzymes responsible for peptidoglycan synthesis (37-39).

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Background

Table 2.1 History of *H. influenzae*

Year	Historical events
1892	*H. influenzae* was first described by Robert Pfeiffer, who isolated it from lung and sputum of patients during influenza pandemic and assumed it is the causative agent of what is now known to be viral influenza.
1931	*H. influenzae* was classified according to the presence of six distinct polysaccharide capsules.
1933	The association of protection against *H. influenzae* meningitis with the level of bactericidal activity of blood was recognized by Fothergill and Wright.
1980	How to conjugate protein carrier molecules to *H. influenzae* capsular polysaccharide (polyribosyl ribitol phosphate; PRP) was reported by Porter Anderson, John Robbins and Rachel Schneerson.
1995	DNA sequence analysis of whole genome of *H. influenzae* standard reference isolate Rd was reported (40).

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Taxonomy of *H. influenzae*

H. influenzae is classified in the Phylum *Proteobacteria*, class Gammaproteobacteria, order Pasteurellales, family Pasteurellaceae, genus *Haemophilus* and species *influenzae*. Fig. 2.1 shows the relationship of 16S phylogenetic tree between members of genus *Haemophilus* compared to other related *Pasteurellaceae*. The closely related bacteria include *H. parainfluenzae*, *H. haemolyticus*, *H. aegyptius*, *H. ducreyi*, *H. segnis*, *H. aphrophilus* and *H. paraphrophilus*.

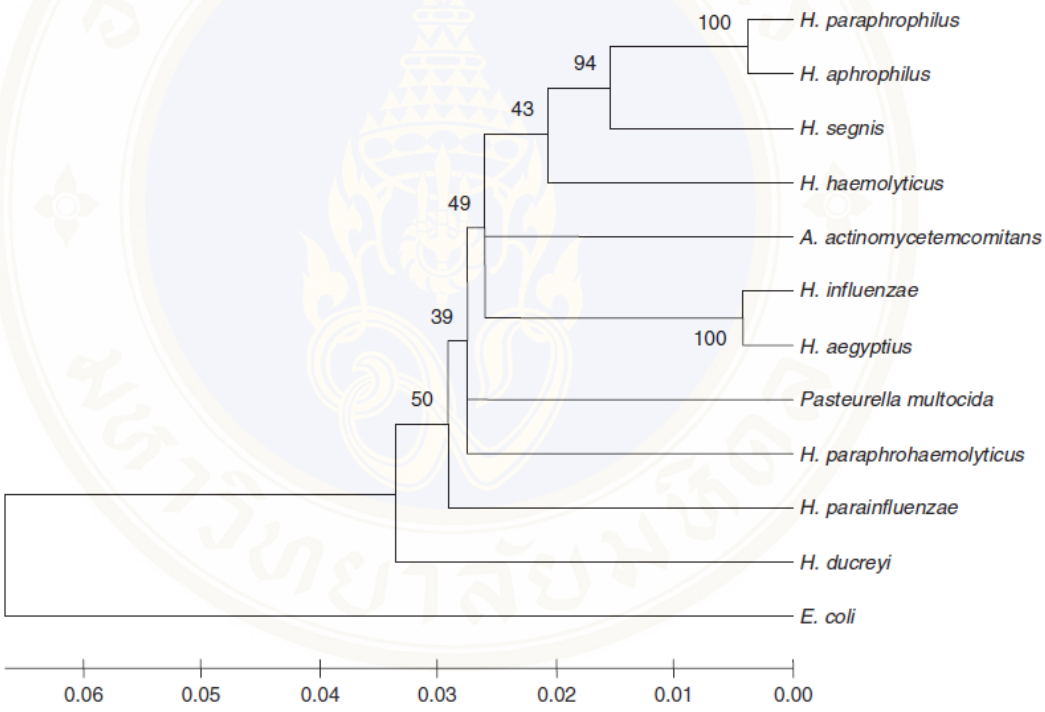

Figure 2.1 Phylogenetic tree of 16S sequences of the main members in genus *Haemophilus* compared to other related *Pasteurellaceae* and rooted on *Escherichia coli* as an out-group

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General characteristics of *H. influenzae*

1. Morphology and structure

H. influenzae is a small (in range 0.2–0.3 to 0.5–0.8 μm) pleomorphic bacteria. It is Gram-negative rod or coccobacillus with occasional longer, filamentous form and non-motile (Fig. 2.2). Some isolates of *H. influenzae* produce a polysaccharide capsule, which is demonstrated in Quellung reaction (swelling of capsule) with type-specific antisera. The most important capsule is type b which is a polymer of ribosyl ribitol phosphate.

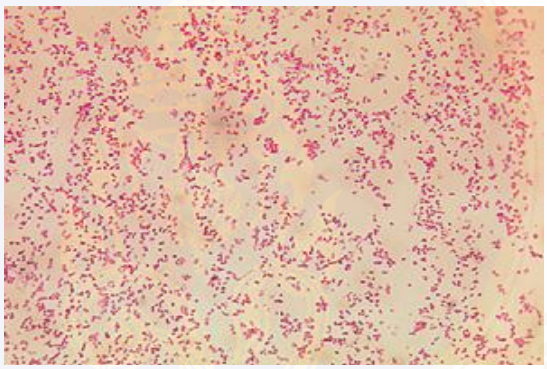

Figure 2.2 *Haemophilus influenzae*: Gram-negative small pleomorphic rods

2. Growth characteristics and culture appearance

The organism grows well on chocolate agar and produces 2–3-μm grey translucent colonies after 18–24 hrs of incubation in 5% CO_2 (Fig. 2.3). It is a fastidious microorganism which its growth depends on a requirement of two factors, named X and V factors:

X factor is hemin, which is required for the synthesis of cytochrome c and other iron-containing respiratory enzymes.

V factor is nicotinamide adenine dinucleotide (NAD), which is essential for oxidation–reduction processes in cell metabolism.

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Both factors are important in identification step, because different species of *Haemophilus* may require either or both of these factors (Table 2.2).

Figure 2.3 *H. influenzae* culture on chocolate agar

Table 2.2 Characteristics of *Haemophilus* species

Species	**Requirement**			**Fermentation**			
	X	V	Hemolysis	Glucose	Sucrose	Lactose	Mannose
H. influenzae	+	+	-	+	-	-	-
H. haemolyticus	+	+	+	+	-	-	-
H. ducreyi	+	-	-	-	-	-	-
H. parainfluenzae	-	+	-	+	+	-	+
H. parahaemolyticus	-	+	+	+	+	-	-
H. segnis	-	+	-	+	+	-	-
H. paraphrophilus	-	+	-	+	+	+	+
H. aphrophilus	-	-	-	+	+	+	+

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3. Population genetics

Capsulated isolates have minority in the population as the majority population occurs in noncapsulated isolates. The evolutionary origins of capsulated isolates are unclear. However; these isolates form a relatively non-diverse population when compared with the much more diverse population in noncapsulated isolates.

4. Virulence factors

H. influenzae possess various kinds of factors that facilitate its pathogenicity in humans (41).

4.1 Capsule

Type b capsule is the most important virulence factor which is associated in pathogenesis of *H. influenzae*. Type b capsule is carried by the majority of *H. influenzae* isolates that cause invasive infections such as meningitis, cellulitis, septic arthritis, bacteremia, and epiglottitis (41). Isolates which carry other types of capsules also cause invasive infections, but their incidence is much lower than type b capsule infections. Type b capsule comprises of a negatively charged phosphodiester-linked linear polymer of disaccharide units of polyribosyl ribitol phosphate (PRP). Capsule protects bacteria from phagocytosis which is the advantage of capsule to bacteria by interference with the binding of complement and opsonizing antibodies to bacterial surface. Moreover, the strong negative charge of polysaccharide capsule may provide electrostatic repulsion to phagocytic cells (42).

4.2 Lipopolysaccharide (LPS)

LPS is a complex glycolipid found on Gram-negative bacterial cell wall which is essential for membrane integrity. In *H. influenzae*, LPS consists of two distinct paths that are lipid A and core oligosaccharide (43). LPS plays an important role in the processes of bacterial colonization, persistence and survival in the human host (44). The lipid A portion is embedded in outer membrane and mediates the endotoxic effects of LPS molecule result in the pathophysiology usually associated

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with severe infection. The host response to lipid A is mediated by activation of Toll-like receptor 4 (TLR4) leading to the binding of LPS-binding protein (LBP) and host cell receptor (CD14) (45, 46). This interaction triggers a cascade of signalling pathway, resulting in the secretion of various mediators and cytokines produced by activated macrophages and monocytes. The overproduction of these effector molecules, such as interleukin-1 and tumour necrosis factor-α provides the pathophysiology of endotoxic shock, which can include hypotension, disseminated intravascular coagulopathy and multi-organ failure. The structure of *H. influenzae* oligosaccharide can be characterized into a conserved inner core region and a variable outer core region. The oligosaccharide can influence interactions of the bacteria with host cells and components of the host immune system.

4.3 IgA Protease

IgA protease is an endopeptidase that inactivates the immunoglobulin IgA1 by enzyme cleavage. The study found nontypeable *H. influenzae* which is isolated from infected patients has the level of IgA1 protease activity higher than isolates which are isolated from asymptomatic carriers (47).

4.4 Pili and other adhesion proteins

H. influenzae possess a various number of adhesins that facilitate the bacterial adherence to epithelial cells and respiratory mucosa, therefore adhesins facilitate the colonization which is the initial event in pathogenesis of *H. influenzae* infection and disease.

Pili (or fimbriae), typically polymeric hair-like structures which extend from bacterial cell surface mediated through the HifE proteins (48). It binds to sialic acid-containing lactosylceramides on cell surfaces (49). Not all *H. influenzae* express pili, there is other non-pilus membrane proteins are involved in attachment process. The examples of adhesions that have been identified are:

High molecular weight (HMW) adhesins, which are present only in non-typeable *H. influenzae* (50).

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H. influenzae serotype a (Hia) is a surface adhesin found primarily on nontypeable isolates that do not express HMW. The protein similar to Hsf which is fibrils that found on type b isolates (51).

The serine protease Hap is structurally but not functionally related to IgA protease. The protein self assembles into fibrillar structures that bind to each other, facilitating the formation of bacterial microcolonies (52). In addition, host-mediated inhibition of the autoproteolysis results in retention of the central protease region on the bacterial cell surface, which facilitates adherence of the bacteria to respiratory epithelial cells.

P5 (peptidases) protein has been shown to be a lytic enzyme that cleaves the bacterial peptidoglycan layer to facilitate membrane fusion during infection and mediate adherence to surface proteins on respiratory cells (53). P5 protein involves in antigenic drift during the process of human colonization with *H. influenzae* (54).

4.5 Lipooligosaccharide

H. influenzae lipooligosaccharides (LOS) are lipopolysaccharides which lack O-specific side chain found in enteric Gram-negative bacteria. Lipooligosaccharides have been shown to be virulence in animal models of bacteremia. One LOS modification is ChoP which has been associated with acute otitis media in the chinchilla model (55). Isolates with ChoP expression are commonly found among pharyngeal commensal isolates (56). Another LOS modification is the addition of sialic acid, which may protect bacteria from host immune system through molecular mimicry. Additional modifications are the glycosyl moieties and O-acetylation of LOS result in increased virulence of *H. influenzae* (57). The virulence of *H. influenzae* LOS appears to stimulate an innate immune response in the human host through interactions with Toll-like receptor 4 (TLR4) leading to NF-κB activating pathway (58) result in a variety of inflammatory molecules production such as IL-6, IL-8, TNFα, and MCP-1 (59).

4.6 Phase and antigenic variation

Bacterial pathogens able to adapt to microenvironments change within and between hosts. *H. influenzae* has evolved a mechanism to alter the expression of cell-

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surface proteins, or enzymes required for the biosynthesis of LPS molecule. This mechanism provides considerable flexibility in the patterns of antigens that can be expressed at any given time within a population of *H. influenzae* (60).

4.7 Iron and heme uptake

Iron is a supply for absolute growth requirement in *H. influenzae*. Hgp proteins are involved in utilization of heme from hemoglobin. Study from mutant NTHi isolates which lacking all *hgp* genes showed reduced virulence in chinchilla model of otitis media when compared to wild type (61) and reduced ability to initiate and sustain type b bacteremia in weanling rats (62). The *hux* operon, consisting of three genes, encodes proteins important in binding heme. A mutant Hib isolate lacking *hxuA-C* produced lower bacterial titers during bacteremia, with associated improved survival rates as compared with wild-type isolate (63).

4.8 Other virulence factors

Additional virulence factors of *H. influenzae* include protein D, a highly conserved lipoprotein that compromises the mucociliary escalator in respiratory cells in tissue culture (64) and has been shown to induce otitis media in rat model (65). Furthermore, Sap proteins confer resistance to antimicrobial peptides in respiratory tract, leading to acute otitis media in chinchillas (66). LuxS, involved in quorum-sensing activity of Gram-negative bacteria, also involved in modulate acute otitis media in chinchillas (67).

5. Pathogenicity of *H. influenzae* infection

5.1 *H. influenzae* colonization

The colonization of *H. influenzae* in host's pharynx is the first step in the pathogenesis of any *H. influenzae* infection.

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5.1.1 Colonization of *H. influenzae* type b (Hib)

The prevalence rates between 0.7 to 5.3% were found in pharyngeal colonization with type b *H. influenzae* in non-immune patients which is very rare cases (68). Furthermore, the prevalence rates of Hib carriage among children in day care were 48 and 71% in toddlers and preschool age children, respectively which in infants and adults and school age children were lower rates (69). Therefore colonization with Hib was found in many unimmunized children.

5.1.2. Colonization with nontypeable *H. influenzae* (NTHi)

The prevalence rates of 25 to 77–84% were found in colonization of nontypeable *H. influenzae* in healthy children which is common cases (70, 71-75). There are many risk factors associated with asymptomatic NTHi colonization that are exposure to tobacco smoke (72, 76, 77) and attending day care (78). The risk factors in day care setting are ≥5 classrooms in the center and suboptimal hygiene practices (76).

5.1.3. *H. influenzae* and biofilms

H. influenzae resides in mucosal surfaces as biofilm (79, 80). Several virulence factors in *H. influenzae* also involve in biofilm formation that are hemagglutinating pili (81), type IV PilA (82) and sialylation of lipooligosaccharides (83).

5.2 Patterns of host response

5.2.1 Encapsulated *H. influenzae* infections

5.2.1.1 Diseases caused by encapsulated *H. influenzae*

Type b *H .influenzae* isolates commonly caused epiglottitis, bacteremia, septic arthritis, periorbital and facial cellulitis and pneumonia. The other capsular types, a, c, d, e, and f, also cause occasional invasive infections.

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Prior the introduction of Hib vaccine, study in USA found type b *H. influenzae* was the most common cause of bacterial meningitis in children in USA. Meningitis is a serious diseases which the early symptoms include fever, lethargy and irritability and later symptoms include vomiting, stiff neck and seizures. Children who present symptoms of bacterial meningitis must require immediate medical care to diagnose and treat.

In non-immune persons, *H. influenzae* type b is the predominant cause of epiglottitis. The symptoms are fever, severe respiratory distress from upper airway obstruction.

Facial cellulitis is an infection of infants that is nearly always caused by *H. influenzae* type b. The symptoms are fever and rapidly developing swelling of the skin on the face with redness.

5.1.2.2 Mechanisms of disease caused by encapsulated *H. influenzae*

Type b accesses to blood stream by unknown mechanisms. It also circulates and seeds to various organs such as meninges, skin, pleural spaces and joints. Moreover, type b isolates are also directly seeded to epiglottis and lower respiratory tract from pharynx. The most important virulence factor for invasive *H. influenzae* disease is type b capsule which results in resistance to phagocytosis and decreased susceptibility of bacteria to complement-mediated lysis and opsonization (84). Moreover, the presence of type b capsule also allows *H.influenzae* to survive during bacteremia infection. Other than capsule such as lipooligosaccharides also stimulate inflammatory cytokines such as Il1β and TNFα (85, 86) that results in the symptoms associated with infection.

5.2.2 Non-typeable *H. influenzae* (NTHi) infections

H. influenzae lacking capsular polysaccharide shows different outer membrane protein patterns from encapsulated isolates (87).

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5.2.2.1 Diseases caused by NTHi

The infections caused by nontypeable *H. influenzae* in healthy persons are limited to respiratory tract and include acute otitis media, sinusitis in children and pneumonia, bronchitis in adults. Since the introduction of conjugated *S. pneumoniae* vaccine in children, NTHi has emerged as the major cause of acute otitis media, ranging 30-52% of infections (88). Furthermore, NTHi also causes acute sinusitis (89), community-acquired pneumonia (90, 91), acute exacerbation of chronic obstructive pulmonary disease (92, 93). Furthermore, NTHi isolates rarely cause bacteremia and meningitis.

5.2.2.2 Mechanisms of disease caused by NTHi *H. influenzae*

NTHi *H. influenzae* first interacts to human via adhere to respiratory mucosal cells, leading to asymptomatic colonization of pharynx. Subsequently, it accesses to the middle ear space through the eustachian tube and subsequently begins an inflammatory response to perform acute otitis media (94). Additional, NTHi also accesses to respiratory sinuses through the ostia and causes acute sinusitis. Although NTHi lacks toxins and capsule, they have other virulence factors that may provide the disease (95). Moreover, study the interactions between NTHi and host cells through Toll-like receptors 2 and 4 and platelet-activating factor receptor (PAFR) may initiate intracellular signals through mitogen-activated kinase cascade or by p38 activation (94).

6. Clinical syndrome

6.1 Hib-invasive diseases

All of Hib infections, meningitis is found over 50% of cases. The remaining is pneumonia, epiglottitis, septicemia, cellulitis and septic arthritis.

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6.1.1 Meningitis

Meningitis is often preceded by signs and symptoms of upper respiratory infection such as pharyngitis, sinusitis or otitis media. The mortality rate is 3-6%.

6.1.2 Acute epiglottitis

Hib is one of pathogen causes acute epiglottitis which shows inflamed epiglottis and surrounding tissues obstruct the airway. The onset of disease is sudden, with the symptoms of fever, sore throat, hoarseness, muffled cough, and rapid progression to severe prostration within 24 hrs. The hallmark of this disease is an inflamed, swollen, cherry-red epiglottis that protrudes into the airway and can be visualized on lateral x-rays.

6.1.3 Cellulitis and arthritis

The symptoms caused by Hib cellulitis are tender, reddish-blue swelling in the cheek or periorbital area. Furthermore, fever and moderately toxic state are often present. The infection may follow by upper respiratory infection or otitis media. Joint infection begins with fever. Moreover, bacteremia is often present in both cellulitis and arthritis.

6.2 Nontypeable *H. influenzae* (NTHi)

6.2.1 Otitis media

It is the main disease which is caused by NTHi *H. influenzae* (96) and associated with irritability and inflamed tympanic membrane. Tympanocentesis is the most reliable means of diagnosing and determining the aetiology of otitis media.

6.2.2 Conjunctivitis

It is a high proportion of disease caused by NTHi *H. influenzae* in children. Culture of conjunctival swabs is the major test for diagnosis.

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6.2.3 Sinusitis

It is associated with local features of infection that are local pain, a sense of pressure in head and occasionally visible pus draining from the ostea of sinuses.

6.2.4 Chronic bronchitis and exacerbation of bronchitis

They are also the main disease manifestations associated with NTHi *H. influenzae* in adults.

6.2.5 Severe invasive diseases

These diseases may be caused by NTHi *H. influenzae*. In adults the main manifestation is pneumonia, particularly among the elderly (96). An unusual manifestation is meningitis, usually in association with previous head trauma, particularly skull fracture (97).

7. Laboratory diagnosis

7.1 Culture

Bacterial culture is the most reliable diagnostic test for *H. influenzae* disease which clinical specimen is obtained from the site of infection. *H. influenzae* is fastidious organism and requires suitable nutrient media containing X and V factors for growth. The growth condition is 35ºC with 5% CO_2. In microbiology laboratories chocolate agar is used to culture *H. influenzae* from clinical specimen and automated culture systems to process hemoculture.

7.2 Identification of *H. influenzae*

In microbiology laboratories, *H. influenzae* are characterized as Gram-negative, pleomorphic rod grown on chocolate agar because it requires both X and V factors. Factor V (NAD) requirement is assessed by bacterial growth surrounding NAD-containing disk placed on a bacterial lawn inoculated on nutrient agar plates or

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by satellitism around *Staphylococcus aureus* colonies that secrete NAD (98). Factor X (heme) requirement is assessed by bacterial growth surrounding heme containing disk or by failure of the colonies to elaborate porphyrin.

7.3 Antigen detection

The commercial kits are available to detect capsular polysaccharide (PRP) of type b *H. influenzae* in serum, cerebrospinal fluid, and urine and may be used to confirm type b *H. influenzae* infection. This technique provides accurate but the interpretation is highly dependent on experience of the technician, therefore this test is not widely used in microbiology laboratories.

7.4 Polymerase chain reaction (PCR)

PCR has been used to detect *H. influenzae* DNA in sterile middle ear effusions (99) and blood of patients with meningitis or pneumonia (100) but this technique is not routinely used for the diagnosis of *H. influenzae* infections.

7.5 Serology

As infection with type b *H. influenzae* leads to stimulation of type b capsule antibodies in children >2 years old. However, it requires 1–2 weeks and is not clinically useful in diagnosing an acute infection with *H. influenzae* type b.

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Typing of *H. influenzae*

There are many typing systems which are based on phenotypic or genotypic characteristics. Phenotypic characteristic consists of capsular type, biotype, outer membrane protein type or multilocus enzyme electrophoresis (MLEE) type. For molecular typing systems, they are based on restriction fragment length polymorphisms, polymerase chain reaction and DNA sequences in various regions of *H. influenzae* genome.

1. Phenotype-based typing methods

1.1 Capsular typing

Serotyping of capsule requires specific reagents and skill as well as experience of laboratory technician (101). Therefore, molecular methods have been developed (102) by using PCR amplification of capsular gene regions. One set of primers is used to amplify *bexA* region, which is conserved in all encapsulated *H. influenzae* isolates and required for export the capsule to cell surface. A second set of primers, are used to amplify the type-specific domain of *cap* gene region in typeable isolates.

1.2 Biotyping

Biotype of *H. influenzae* can be classified based on 3 biochemical tests i.e., production of indole, the presence of urease and ornithine decarboxylase activities. Eight biotypes of *H. influenzae* have been identified (Table 2.3). Most clinical isolates are biotypes I, II, or III. Although specific biotypes have been associated with virulence, this technique is not enough to cluster *H. influenzae* for meaningful epidemiologic investigation.

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Table 2.3 Biotypes of *H. influenzae*

Biotypes	**Indole**	**Urease**	**Ornithine decarboxylase**
I	+	+	+
II	+	+	-
III	-	+	-
IV	-	+	+
V	+	-	+
VI	-	-	+
VII	+	-	-
VIII	-	-	-

1.3 Outer membrane typing

This technique used outer membrane protein for subtyping *H. influenzae*, by digestion of bacterial outer membrane with detergent or by sonication and separation of outer membrane fragments by gel electrophoresis. This technique was used to track transmission of *H. influenzae* isolates and to compare commensal isolates with otitis media isolates from the same person (103).

1.4 Multilocus enzyme electrophoresis (MLEE)

It differentiates *H. influenzae* isolates based on the electrophoretic pattern differences of 15–17 metabolic enzymes (104–106). Study of *H. influenzae* electrophoretic types among encapsulated isolates from 280 types found type b isolates fall into two major phylogenetic groups, division I and division II (104). Since non-typeable isolates are phylogenetically distinct from type b isolates and more heterogeneous than type b isolates. This technique is costly and time consuming to perform and has largely been abandoned.

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2. Molecular-based genotyping methods

According to capsular typing and biotyping, which are limited by their ability to identify only seven serotypes (including nontypeable) and eight biotypes. Therefore, molecular typing systems have been developed to expand the ability to group *H. influenzae*.

Each molecular typing method is based on various genetic alterations which provide the increasing in polymorphisms of *H. influenzae* genome and can distinguish isolates by characterization of different molecular parameters (Table 2.4). Therefore, isolates grouped as similar by one technique may not be grouped as similar by another technique.

Table 2.4 Molecular-based typing methods of *H. influenzae*

Methods	**Discriminator**	**Typing based on**
Restriction fragment length polymorphism (RFLP)		
Pulsed-field gel electrophoresis (PFGE)	Sizes of restriction fragments	Patterns of bands on gel
Pulsed-field gel electrophoresis (PFGE) +hybridization	Sizes of restriction fragments that hybridized to gene-specific probes	Patterns of bands on gel
Polymerase chain reaction (PCR)		
Random amplified polymorphic DNA (RAPD), Enterobacterial repetitive intergenic consensus (ERIC), Repetitive sequence PCR (RepPCR)	Sizes of PCR-generated amplicons	Patterns of bands on gel
DNA sequence		
Multilocus sequence typing (MLST), Multilocus variable number tandem repeat analysis (MLVA), 16SDNA	Nucleotide sequences of PCR-generated amplicons	Nucleotide sequence differences

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2.1 Restriction fragment length polymorphism (RFLP)-based typing methods

2.1.1 Ribotyping

Ribotyping can distinguish isolates of bacteria by restriction length polymorphisms of their genetic regions that contain genes-encoding ribosomal RNA. The bacterial DNA is digested with frequent-cutting restriction endonucleases, then the resulting bands are separated by gel electrophoresis, and the gels are investigated with a labeled rRNA probe (107). For example, a study from Taiwan, ribotyping yielded 6 patterns among 55 type b isolates, 9 patterns among 10 non-b-typeable isolates, and 12 patterns among 22 non-typeable *H. influenzae* (108). This technique is useful in genotyping *H. influenzae* but less discriminatory than pulsed-field gel electrophoresis (109).

2.1.2 Pulsed-field gel electrophoresis (PFGE) typing

PFGE is often considered the gold standard of molecular genotyping. It distinguishes bacterial isolates by using difference in sizes of the restriction fragments. The DNA is digested with rare-cutting (8 base pair) restriction endonucleases and the resulting bands are separated by gel electrophoresis. The differences in *H. influenzae* can be examined by the DNA banding patterns. The usage of PFGE was reported to document the relationships for *H. influenzae* isolated from children attending the same day care (70, 110); for isolates from the same individual (111); for isolates from household contacts of patients with invasive *H. influenzae* infections (112, 113) and for non-beta-lactamase-resistant *H. influenzae* (114, 115). This technique shows the highly diverse phylogenetic structure of *H. influenzae*, particularly in non-typeable isolates.

2.2 Polymerase chain reaction (PCR) fragments-based typing methods

PCR-based methods rely on analysis of DNA fragments derived from amplification of specific bacterial genome regions.

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2.2.1 Random amplified sequence-based genome typing

Random amplified sequence typing or RAPDTM is a PCR-based method in which amplicons are generated by using nucleic acid primers. In many studies, RAPD results give labile with poor reproducibility on repeated testing of the same sample (112, 116, 117) although commercial kits may help to reduce the poor reproducibility to some extent (118).

2.2.2 Enterobacterial repetitive intergenic consensus (ERIC) typing

ERIC is a PCR-based technique in which amplicons are generated by using nucleic acid region primers that hybridize with many gene regions in *E. coli*. These regions are absent in *H. influenzae*, thus when used to type *H. influenzae*, this technique depends on amplification of random sequences. The poor reproducibility of random amplified sequence typing may be related to the low stringency required of the amplification reaction to allow hybridization of the random primers (109).

2.2.3 Repetitive element PCR typing

It uses PCR in order to amplify DNA regions located between repeated sequences in bacterial genomes.

2.3 Sequence-based genome typing methods

2.3.1 16S rRNA typing

The sequence differences in 16S ribosomal RNA genes are basis for genotyping in many pathogenic bacteria, including *H. influenzae* (119). The latter has six 16S rRNA operons which may not be identical in sequence (40), potentially complicating sequence analysis of isolates possessing differing 16S rRNA genes (120).

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2.3.2 Multilocus sequence typing (MLST)

MLST is an epidemiologic tool and has been widely used to genotype a large number of bacterial species (121). The principle of this method is based on indexing variation in the slowly evolving core genes (122). In *H. influenzae*, this technique uses sequences of seven genes that encode central metabolic enzymes or housekeeping genes which are *adk*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi*, and *recA*. The sequence types (ST) of the isolates are defined on the basis of their allelic profile over the seven loci examined.

2.3.3 Multilocus variable number tandem repeat analysis (MLVA)

Tandem repeat regions possessing variable numbers of repeats have been identified in almost all bacterial species. The number of repeats in each repeat locus varies from isolate to isolate because of genetic instability of the repeat regions (123) and forms the basis for MLVA genotyping. This technique was found to be more discriminating for differences in type b *H. influenzae* than was MLST (124).

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Epidemiology

1. Type b *H. influenzae*

Type b *H. influenzae* (Hib) is a major cause of childhood infectious disease. The carriage rates vary after birth, and risk factors for carriage are advancing age from birth until 4 years, underdeveloped country, crowding, day-care attendance and presence of siblings. The prevention is Hib immunization which soon eliminates carriage and produces a marked herd effect protecting against disease (125). As disease caused by Hib has been near eliminated by the highly efficient conjugate Hib vaccine (126). The epidemiology of disease which deals with either pre-vaccine era or where vaccine has not been nationally implemented found 60% of the world's children are unimmunized and unprotected (126). The major burden of disease caused by Hib is meningitis which varies among countries or regions in the world. In USA, before implementation of vaccine; it was the most important cause of childhood meningitis accounting for 80% of cases (127, 128). There are many factors that affect the epidemiology of disease. For example, age factor; disease peaks at 9 months of age and declines to very low levels by 4 years, male gender, moreover, its incidence varies by country and ethnic origin.

Implementation of Hib-conjugate vaccine has the largest impact on the epidemiology of disease. It has nearly disappeared from North America and Europe. There has been a similar dramatic decline in Gambia, a resource-poor country with a very high incidence before the implementation of a national immunization programme (126).

2. Nontypeable *H. influenzae*

Nontypeable *H. influenzae* is acquired after birth, and 20–80% of people carry this organism in all ages. Nontypeable *H. influenzae* causes otitis media, sinusitis, chronic bronchitis and pneumonia (96) which otitis media is one of the most common childhood diseases. The other diseases are associated with adults as chronic bronchitis and pneumonia occur more frequently with advancing age.

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Treatment of *H. influenzae* infections

The first-line antibiotic treatment for Hib-invasive disease is third generation cephalosporin. Ceftriaxone and cefotaxime are effective for treating *H. influenzae* meningitis and septicemia. Moreover, adjunctive steroid therapy can reduce rate of deafness in children with Hib meningitis. In poor countries, chloramphenicol alone or combination with ampicillin is effective for therapy.

For noninvasive infections such as otitis media, sinusitis and chronic bronchitis, the antibiotic treatment is much less clear.

Control and prevention of *H. influenzae* infections

1. Prevention of type b infections

1.1 Hib vaccines

Polyribosyl ribitol phosphate (PRP), the polysaccharide of type b *H. influenzae*, has the important properties in vaccine antigen. PRP was the first purified polysaccharide formulation used as *H. influenzae* vaccine and was licensed in 1985. The study in this vaccine which consisted only the purified PRP found children under age 2 years developed low titers of serum antibodies that was insufficient to protect against invasive disease (129) because it waned quickly and there was no immune memory (130). As the polysaccharides conjugated to proteins can enhance immune response and protective immunity in animals, therefore vaccines with purified PRP conjugated to a variety of protein carriers were developed (131). Table 2.5 shows the Hib conjugate vaccines can differ in immunogenicity which may be related to the nature of carrier protein and the method of protein coupling to polysaccharide.

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Table 2.5 Hib conjugate vaccines

Vaccine	Protein carrier	Trade name	Age groups	Antibody response in infancy
PRP-D	Diphtheria toxoid	ProHIBiT	15-59 months	Moderate after second dose
HbOC	CRM 197 mutant	HibTITER	2, 4, 6, and 12-15 months	Good after second dose
PRP-OMP	*N. meningitidis* outer membrane protein	PedvaxHIB	2, 4, and 12-15 months	Moderate after first dose
PRP-T	Tetanus toxoid	ActHIB	2, 4, 6, and 12-15 months	Good after second dose
PRP-T/DTaP	Tetanus toxoid	TriHIBit	Licensed for fourth dose only	Not applicable
PRP-OMP/Hepatitis B	*N. meningitidis* outer membrane protein	Recombivax	2, 4, 6, and 12-15 months	Moderate after first dose

PRP-D is no longer used in young infants because of its poor immunogenicity and poor protection against Hib infection studied in Alaskan native children where a high level of natural disease in the population (132). In case of PRT-T, PRP-OMP and HbOC shows the high levels of anti-PRP antibodies in infants and is highly protective against Hib invasive infection (133).

1.1.1 Use of current vaccines

The dose of each Hib conjugate vaccine is 0.5 ml injection intramuscularly. In USA, the recommended schedule is for all children to receive the

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primary vaccine series at age 2-4 months for PRP-OMP or 2, 4, and 6 months for PRPT and booster at age 12–15 months. Moreover, Hib vaccines can be administered separately or combination with other vaccines (134). Two conjugated vaccines are available as combination vaccines such as PRP-T/DTaP and PRP-OMP/hepatitis B. All Hib vaccines are safe but there may be a local reaction in some children.

1.1.2 Impact of Hib vaccines

The widely use of Hib vaccine in USA resulted in the dramatic reduction in the number of invasive Hib cases (135). Fig. 2.4 shows the reduction in cases of Hib meningitis relative to licensure dates and ages of Hib vaccine usage. Fig. 2.5 shows maintained low rates of *H. influenzae* invasive diseases since the introduction of vaccines. In spite of the usage of vaccine, however, there are some children remain under-vaccinated. Furthermore, the mortality rates of Hib meningitis declined rapidly after the introduction of PRP-based vaccines (136). Prior to introduction of vaccines, mortality rates were 1.5 deaths per 100,000 among children 1–4 years old, then in the year following use of conjugate vaccines fell to 0.11/100,000 in children ≥2 months old. In addition, decreased incidence and mortality rates of Hib infection may be related to the role of conjugate vaccines in reducing colonization of *H. influenzae* type b (137).

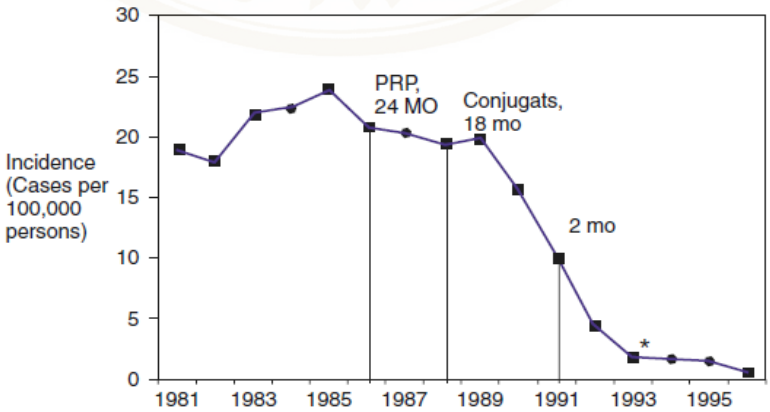

Figure 2.4 Hib meningitis in children less than 5 years old according to the National Bacterial Meningitis Reporting System in 1980–1991

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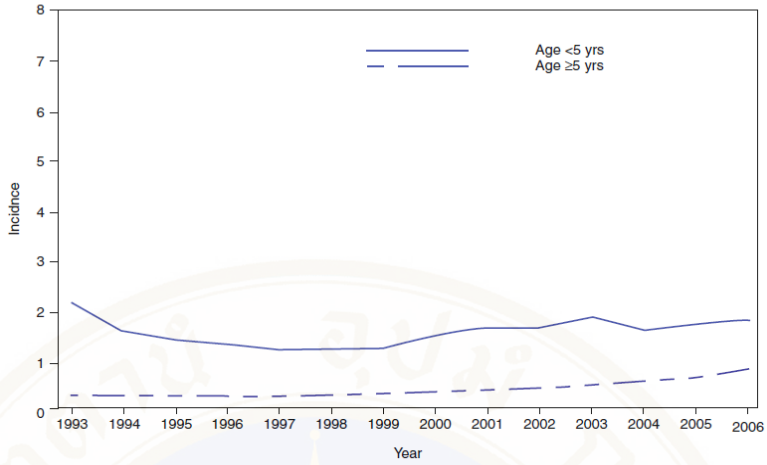

Figure 2.5 The incidence of *H. influenzae* invasive disease according to age group in USA, 1992–2005

1.2 Vaccines against NTHi

Because NTHi lacks capsular polysaccharide, so Hib vaccines do not protect against infections caused by NTHi. The effort to produce vaccine against NTHi was firstly involved identification of bacterial surface antigens (138) which the candidate antigens were adhesions P5, high molecular weight protein (OMP 26, P6) and detoxified lipooligosaccharide. A combination vaccine is composed of 11 different *S. pneumoniae* polysaccharides conjugated to protein D, a cell surface lipoprotein of NTHi, as a carrier protein. A combination vaccine showed only limited efficacy in preventing NTHi acute otitis media (139). Since NTHi primarily causes respiratory tract infections, novel approaches to vaccine development e.g., direct mucosal application or modulation of specific immune response, may be required for a successful NTHi vaccine (140).

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Mechanisms of antimicrobial resistance

In general, susceptibility or resistance to beta-lactams in bacteria is involved in interaction between permeability of bacterial cell to antibiotic, the presence, spectrum of activity and production of beta-lactamases, the presence and efficiency of efflux mechanisms and affinity of antibiotic binding to penicillin-binding protein (PBP) target site (141).

Macrolides and beta-lactams have been the most commonly prescribed antibiotic used to treat infections with *H. influenzae*. However, it is difficult to find good macrolides due to the expression of bacterial efflux pump (142, 143) which plays an important role in efflux of erythromycin, azithromycin, clarithromycin and new ketolides.

In *H. influenzae*, the resistance to beta-lactams is emerged in the early 1970s and rate of resistance has increased since that time. *H. influenzae* can acquire ampicillin resistance through two different mechanisms. The first one is the production of beta-lactamases, which are usually referred to TEM-1 (36) and ROB-1 (13), and is found in beta-lactamase positive, ampicillin-resistant isolates (BLPAR). Another mechanism is a conformational change in penicillin-binding proteins (PBPs) which is found in beta-lactamase negative, ampicillin-resistant isolates (BLNAR). PBPs are enzymes necessary for cell wall synthesis, which results in reduced affinity to ampicillin (37-39). A very small ratio of isolates possesses both beta-lactamase production and PBPs alteration mechanisms which are found in beta-lactamase positive, amoxicillin/clavulanic acid-resistant isolates (BLPACR) (4-7).

Susceptibility to all beta-lactams in *H. influenzae* is generally predicted by susceptibility to ampicillin as defined by CLSI minimal inhibitory concentration (MIC) breakpoints (8) as shown in Table 2.6.

Table 2.6 Susceptibility to all beta-lactams in *H. influenzae* according to CLSI (8)

	Susceptible	**Intermediate**	**Resistant**
MIC	≤1 µg/ml	2 µg/ml	≥4 µg/ml

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1. Beta-lactamases

Beta-lactams are a family of antimicrobial agents consisting of four major groups which are penicillins, cephalosporins, monobactams and carbapenems grouped together based on a shared structural feature, the beta-lactam ring as shown in Table 2.7 (8, 144). They act on bacteria by inhibiting enzymes that catalyze the cross-linking reactions of D-alanyl peptides or inhibit transpeptidation reaction on peptidoglycan strands of the growing cell wall which, result in bacterial cell death. Furthermore, autolysins, bacterial enzymes that lyse cell wall are activated. Beta-lactams inhibit not just a single enzyme involved in cell wall synthesis, but also a family of related enzymes (4-8) in different bacteria. To bypass the action of these antimicrobial mechanisms, bacteria resist by producing beta-lactam inactivating enzymes (beta-lactamases), mutations in penicillin binding proteins and alteration in porin channels limits access to transpeptidases in Gram-negative bacteria. The beta-lactams are the most common treatment for bacterial infections. The rates of bacterial resistance to antimicrobial agents are also increasing worldwide (144).

The major mechanism of beta-lactam resistance is beta-lactamase production. Beta-lactamases disrupt the amide bond of the four-membered beta-lactam ring, leading to ineffective compound (145, 146). Interestingly, beta-lactamases are structurally related to penicillin-binding proteins (PBPs) and may have evolved from these beta-lactam-binding enzymes (145, 146). Four molecular classes of beta-lactamases are shown in Table 2.8.

Action of beta-lactamase, firstly, the enzyme associates non-covalently with antibiotic. Then beta-lactam ring is attacked by free hydroxyl on side chain of a serine residue at the active site of enzyme, yielding a covalent acyl ester. Finally, hydrolysis of ester makes active enzyme and inactive drug (Fig. 2.6). This mechanism occurs in beta-lactamases classes A, C and D, while class B utilizes a zinc ion to attack beta-lactam ring (147).

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Table 2.7 Groups and examples of beta-lactam antimicrobial agents

Groups	**Examples of antimicrobial agents**
Penicillin	Penicillin G
	Penicillinase-resistant penicillins: methicillin, nafcillin, oxacillin, cloxacillin
	Aminopenicillins: ampicillin, amoxicillin
	Carboxypenicillins: carbenicillin, ticarcillin
	Ureidopenicillins: azlocillin, mezlocillin, piperacillin
Cephalosporin	First generation: cefazolin, cephalothin, cephalexin
	Second generation: cefuroxime, cefaclor, cefamandole, cephamycins (cefotetan, cefoxitin)
	Third generation: cefotaxime, ceftriaxone, cefpodoxime, ceftizoxime, cefoperazone, ceftazidime
	Fourth generation: cefepime, cefpirome
Carbapenem	Imipenem, meropenem, ertapenem
Monobactam	Aztreonam

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Table 2.8 Classification and properties of beta-lactamases

Ambler classifi-cation	**Type of enzymes**	**Preferred substrates**	**Inhibited by:**		**Representative enzymes**
			CA	**EDTA**	
A	Penicillinase	Penicillins	+	-	Penicillinases from Gram-positive bacteria
	Restricted-spectrum beta-lactamase	Penicillins, cephalosporins	+	-	TEM-1, TEM-2, SHV-1
	Extended-spectrum bata-lactamase	Penicillins, narrow and extended-spectrum cephalosporins, monobactams	+	-	Numerous SHV and TEM variants, CTX-M-1 to CTX-M-28, PER-1 & PER-2, VEB-1, GES-1, IBC-1, several chromosomal enzymes in Gram-negative bacteria
	Inhibitor-resistant beta-lactamase	Penicillins, cephalosporins	-	-	TEM-30 to -41, -44, -45, -51, -54
	Inhibitor-resistant extended-spectrum beta-lactamase	Penicillins, narrow and extended-spectrum cephalosporins (low level)	-	-	TEM-50, -68,-80
	Carbapenemase	Penicillins, cephalosporins, carbapenems, monobactams; sometimes extended-spectrum bata-lactams	+	-	NMC-A, SME-1 to SME-3, IMI-1, KPC-1 to KPC-3, GES-2, SHV-38

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Table 2.8 Classification and properties of beta-lactamases

Ambler classification	**Type of enzymes**	**Preferred substrates**	**Inhibited by:**		**Representative enzymes**
			CA	**EDTA**	
B	Carbapenemase	Most beta-lactams, including carbapenems and extended-spectrum beta-lactams and 4th generation cepharosporins	-	+	IMP-1 to IMP-13, VIM-1 to VIM-7, SPM-1, several chromosomal enzymes of Gram-negative bacteria
C	Expanded-spectrum cephalosporinase	Penicillins, narrow and extended-spectrum cephalosporins, cephamycins, monobactams	-	-	CMY-2 to CMY -13, LAT-1, MOX-1 and MOX -2, FOX-1 to FOX-6, ACT-1, MIR-1, DHA-1 and DHA-2, ACC-1, CFE-1, several chromosomal enzymes of Gram-negative bacteria
D	Narrow-spectrum penicillinase	Penicillins, cloxacillin	±	-	Numerous OXA variants
	Extended-spectrum beta-lactamase	Penicillins, cloxacillin, extended-spectrum beta-lactams, sometimes monobactams or 4th generation cephalosporins	±	-	Several OXA-2 and OXA-10 derivatives, OXA-18, OXA-29, OXA-30, OXA-31, OXA-32, OXA-45

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Table 2.8 Classification and properties of beta-lactamases

Ambler classifi-cation	Type of enzymes	Preferred substrates	Inhibited by:		Representative enzymes
			CA	EDTA	
D	Carbapenemase	Penicillins, oxacillin, carbapenems	+	-	OXA-23 to OXA-27, OXA-40, OXA-48, OXA-54

CA, clavulanate; EDTA, Ethylenediaminetetraacetic acid

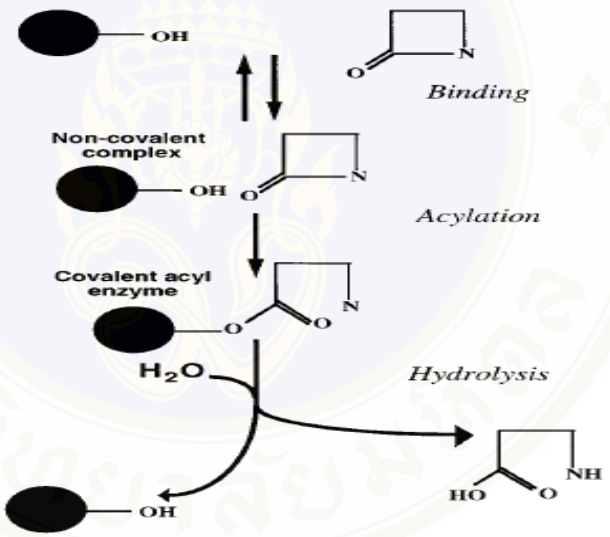

Figure 2.6 Action of serine beta-lactamase

Study in a global survey of over 2,000 beta-lactamase-positive isolates, the prevalence of TEM-1 type beta-lactamase in 93.7% and ROB-1 type beta-lactamase in 4.6% was reported (13, 14, 148). Both TEM and ROB-1 type are plasmid-mediated class A serine beta-lactamases which confer resistance to ampicillin and are inhibited by beta-lactamase inhibitors (149-152).

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1.1 TEM beta-lactamases

TEM-1 and TEM-2 beta-lactamases are common in Gram-negative bacteria, particularly Enterobacteriaceae, which are encoded by bla_{TEM} genes. The bla_{TEM} in *H. influenzae* was transposed on Tn*2* or Tn*3* from Enterobacteriaceae onto cryptic plasmids (153-156). Most evidence indicates that predominate type of TEM beta-lactamase in *H. influenzae* is TEM-1 (11, 148). *H. influenzae* have two groups of TEM-specifying plasmids. The small (<10 kb) nonconjugative plasmids carry bla_{TEM}, whereas the larger (40 kb) conjugative plasmids often carry other resistance genes e.g., chloramphenicol, tetracycline or kanamycin resistance (155, 157-159). The larger plasmids are more common and more considerable in dissemination of bla_{TEM} genes in *H. influenzae* (159, 160).The study found C32T mutation in bla_{TEM-1} which encodes P3 to Pa/Pb promoters is important to increase the amount of enzyme produced and confer ampicillin resistance (154).

1.2 ROB beta-lactamase

ROB-1 beta-lactamase was first described in 1981 in a clinical isolate of Hib which causing meningitis (161). Later it was also detected in a number of related organisms e.g., *Pasteurella multocida* and *H. ducreyi* (36, 148, 162-165). The study on chromosomal ROB-1 gene in *P. aerogenes* found this gene might carry on transposable element. Moreover, the presence of small cryptic plasmids in many animal isolates of *P. multocida* suggests small ROB-1-specifying plasmids might increase from insertion of transposable element into these cryptic plasmids (162). When ROB-1 was detected, the presence of beta-lactamase was initially missed because the negative result of nitrocefin test. Then the organism was found to be ampicillin resistant and nitrocefin test was positive when repeated with a very heavy inoculum (161). The MIC data of TEM-1, ROB-1 producing and beta-lactamase negative *H. influenzae* isolates is shown in Table 2.9.

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Table 2.9 MIC data for TEM-1, ROB-1producing and beta-lactamase negative *H.influenzae* isolates

Drugs	**Minimal inhibitory concentration result (µg/ml) for isolate type:**								
	TEM-1			ROB-1			beta-lactamase negative		
	50%	90%	Range	50%	90%	Range	50%	90%	Range
Ampicillin	≥32	≥32	2-≥32	≥32	≥32	8-≥32	0.25	0.5	0.12-≥32
Amoxicillin-clavulanate	1	2	0.12-≥8	0.5	1	0.25-2	0.5	1	0.12-≥8
Cefaclor	4	16	0.5-≥128	16	64	2-≥128	2	8	0.5-≥128
Cefpodoxime	0.06	0.12	≤0.015-2	0.06	0.06	≤0.015-0.25	0.06	0.12	≤0.015-4

Beta-lactamase producing is a major mechanism in beta-lactam resistance in *H. influenzae* isolates in almost all areas.

2. Beta-lactamase negative, ampicillin resistant (BLNAR) *H. influenzae*

2.1 Characteristic of (BLNAR) *H. influenzae* isolates

BLNAR isolate means the isolate which must be beta-lactamase negative and has ampicillin MIC above CLSI resistant breakpoint of ≥4 µg/ml and isolates with an MIC of 2 µg/ml is interpreted as intermediate, but it is often contain in the BLNAR category (19). Moreover, the use of PCR and DNA sequencing in order to detect specific mutations in the *ftsI* gene, can categorize BLNAR isolates as low and high BLNAR or BLNAR.

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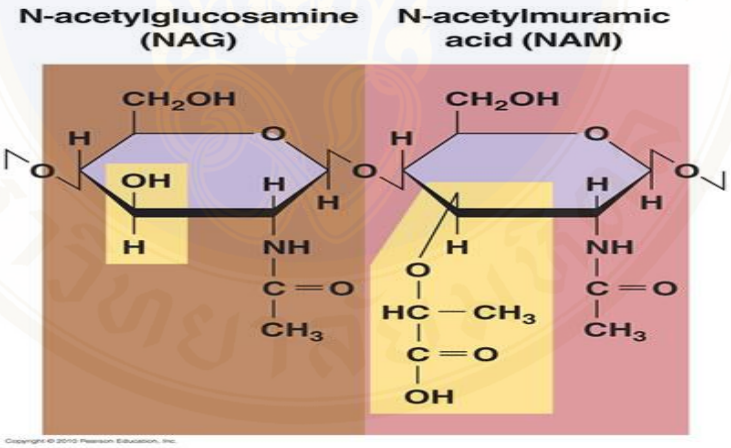
2.2 The mechanism of ampicillin resistance in (BLNAR) *H. influenzae* isolates

Penicillin and other beta-lactam antibiotics target to several bacterial enzymes (penicillin-binding proteins or PBPs). PBPs are necessary for growth and maintenance peptidoglycan layer, which is a part of bacterial cell wall and also protects the cell from osmotic stress. Therefore, inhibition of peptidoglycan biosynthesis leading to inhibition of cell growth. The mechanism of BLNAR *H. influenzae* resistance to beta-lactams occurs via altered PBPs.

2.2.1 Bacterial peptidoglycan

It consists of a backbone chain of repeating two-sugar units, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) (Fig. 2.7).



(http://classes.midlandstech.edu/carterp/Courses/bio225/chap04/lecture4.htm)

Figure 2.7 Structure of N-acetylglucosamine and N-acetylmuramic acid

The biosynthesis of peptidoglycan involves approximately 30 bacterial enzymes. The first step is precursor formation which occurs in cytoplasm. The last step is completion of cross-link between peptidoglycan chains. This occurs via transpeptidation reaction. The transpeptidase is a membrane-bound. The terminal

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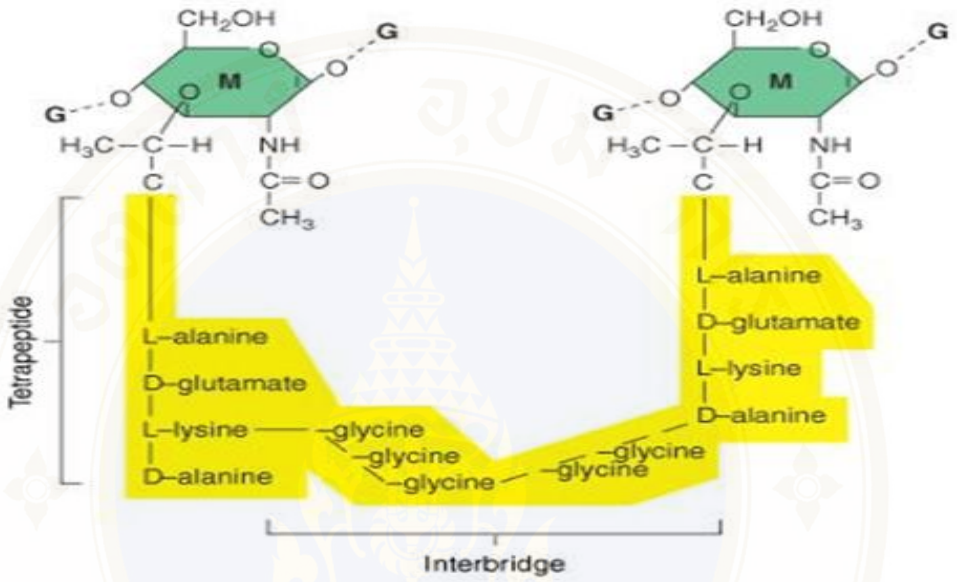
glycine residue in pentaglycine bridge is linked to the fourth residue of the pentapeptide (D-alanine) leading to releasing the fifth residue (D-alanine) (Fig. 2.8). The last step of peptidoglycan synthesis is inhibited by beta-lactams.



(http://www.pc.maricopa.edu/Biology/rcotter/BIO%20205/LessonBuilders/Chapter%204%20LB/Ch4Lessonbuilder6.html#cell envelope (p.94-99)

Figure 2.8 Transpeptidase reaction

2.2.2 Penicillin-binding proteins (PBPs)

PBPs (bifunctional enzymes) contain glycosyltransferase and transpeptidase domains (170). It catalyzes the polymerization of glycan strand via transglycosylation reaction and cross-linking between glycan chains via transpeptidation reaction. Penicillin-binding protein is the target to beta-lactam antibiotics because beta-lactam antibiotics are structurally analogous to D-Ala-D-Ala (Fig. 2.9).

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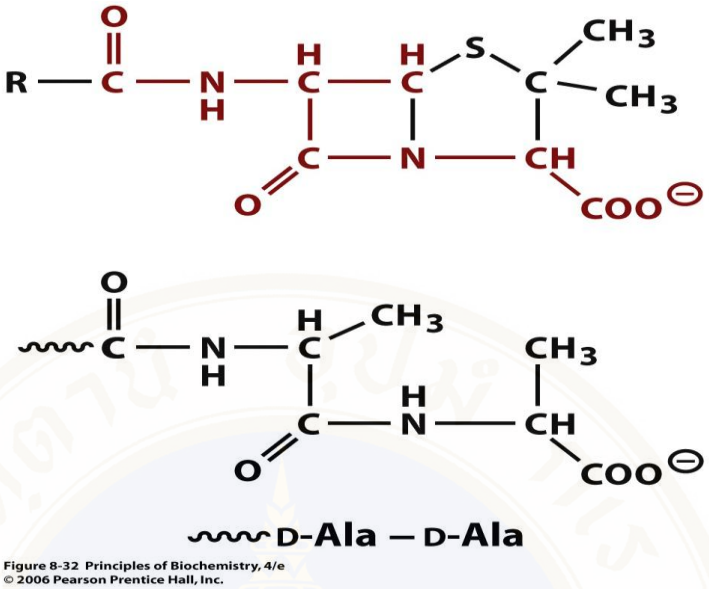

(http://sandwalk.blogspot.com/2007/06/how-penicillin-works-to-kill-bacteria.html)

Figure 2.9 The structure of beta-lactams is shown on top and structure of D-Ala-D-Ala

Altered in PBPs which reduced affinity to beta-lactam antibiotics are more commonly found in Gram-positive than Gram-negative bacteria and high resistance usually occurs in several steps rather than single-step mutation.

Bacteria have a variable number of PBPs (Table 2.10). They are divided into two main categories i.e., high and low molecular mass PBPs. The C-terminal penicillin binding domain of both classes has a transpeptidase activity which catalyzes peptide cross-linking between two adjacent glycan chains.

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Table 2.10 PBP-associated resistance in bacteria

Bacteria	Resistant to	PBP (alteration)
S. aureus	Methicillin, most beta-lactams	2a (decreased binding)
	Cephalexin	3 (decreased binding)
S. epidermidis	Methicillin, most beta-lactams	2a (decreased binding)
S. pneumoniae	Ampicillin	1a, 2x, 2a, 2b (decreased binding)
E. faecalis	Most beta-lactams	1, 3 (decreased binding)
	Most beta-lactams	3 (overproduced)
E. faecium	Most beta-lactams	1, 2 (decreased binding)
E. coli	Mecillinam	2 (overproduced)
	Cephalexin	3 (decreased binding)
P. aeruginosa	Piperacillin	3 (decreased binding)
	Cefsulodin	3 (decreased binding)
H. influenzae	Beta-lactams	3, 4, 5, (decreased binding)
A. calcoaceticus	Beta-lactams	1, 3 (decreased binding)
N. gonorrhoeae	Beta-lactams	1,2 (decreased binding)
N. meningitidis	Beta-lactams	2 (decreased binding)
B. fragilis	Cefoxitin	1 (decreased binding)
	Cefoxitin	1, 2 (decreased binding)

2.2.3 PBPs in *H. influenzae*

H. influenzae contains 8 PBPs which are PBP1-PBP8 according to decreased molecular weight (Table 2.11) (171). Later, PBPs were referred as PBPs IA, IB, 2, 3A, 3B, 4, 5 and 6, respectively, based on binding capacities of various proteins for different beta-lactams similar to PBPs in *Escherichia coli* (30, 173). Table 12.2 shows BLNAR *H. influenzae* discovery.

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Table 2.11 Properties of PBPs in *H. influenzae*

PBPs	Approximately molecular weight (Da)	[^{14}C] penicillin G bound (%)
1	90,000	15.6
2	84,000	4.8
3	75,000	7.9
4	68,000	8.6
5	64,000	13.8
6	48,000	10.6
7	41,000	27.0
8	27,000	

Table 2.12 Discovery of BLNAR *H. influenzae*

Year	Historical events
1974	Ampicillin resistance in beta-lactamase-negative isolates of *H. influenzae* was first reported (174).
1980	Ampicillin-resistant Hib isolate from endocarditis and meningitis cases in which the presence of beta-lactamase was not shown, but an alteration to the target site was proposed as hypothesis (175).
1984	Genomic DNA from BLNAR Hib clinical isolate was able to transform to susceptible *H. influenzae* Rd isolate demonstrated reduced binding affinity of PBP3A and PBP3B (176). Thus, alterations in PBP3A and PBP3B were established as resistance mechanism in BLNAR (37, 177-178).

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2.2.4 Alteration in PBPs causing antibiotic resistance in *H. influenzae*

The mechanism causing BLNAR was observed in Canadian isolates which were separated into 3 groups based on ampicillin MICs of 0.5-1, 2-4, and ≥8 μg/ml for groups I, II, and III, respectively. In addition, genomic DNA from these isolates was transformed to *H. influenzae* susceptible isolate, which the transformants showed the same level of ampicillin resistance and decreased binding affinity of PBP3A and PBP3B when compared with donors. While examination of outer membrane proteins, porin function and lipopolysaccharide profiles in donor transformants could not explain differences in ampicillin resistant levels. Level of resistance in BLNAR isolates may relate to PBP modification. Expanded cell filamentation was also shown in group III transformant, indicating a high degree of septal dysfunction, which is compatible with alterations in PBP3 (177). Subsequently, PBP profiles of 25 Japanese BLNAR isolates were analyzed in 2001 and found the binding affinity of PBP3A was decreased in all isolates and in some isolates for PBP3B and PBP4. In addition, mutations in the *ftsI* gene encoded PBP3, but not the *dacB* gene encoded PBP4, related to ampicillin resistance (179). Later study, sequencing *ftsI*, *dacB*, and *dacA* (PBP 5) genes of cefuroxime-resistant isolates found no evidence of association between *dacA* or *dacB* mutations and cefuroxime resistance, but found evidence supporting the role of *ftsI* mutations (24).

The *ftsI* gene encodes transpeptidase region of PBP3 in location of 1,197,840-1,199,672 nucleotide from 1,830,138 nucleotide of *H. influenzae* Rd KW20, complete genome (Fig. 2.10). Genes and their products for resistance mechanism in *H. influenzae* are shown in Table 2.13. Most PBPs have three highly conserved amino acid motifs which are essential for their function (180). In *H. influenzae* PBP3, these motifs are S327-T-V-K, S379-S-N, and 513-T-G (179). A study from Ubukata et al found many amino acid substitutions appeared in vicinity of conserved PBP3 motifs (179). The usage of three-dimensional crystallographic structure of PBP2X in *S. pneumoniae* as a guide found the substitutions near the active site pocket that surrounded by conserved motifs (24). Furthermore, examination of cefuroxime-resistant isolates from the United Kingdom by using computer modeling, based on three-dimensional structure of *S. pneumoniae* PBP2x in order to examine

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how S357N substitution would affect interaction of cefuroxime with PBP3 (179). It was found that asparagine side chain produced steric hindrance with the adjacent valine at position 362. This may alter orientation of V362-P366 loop and this loop is adjacent to active site of PBP, therefore change in orientation of PBP may limit access to beta-lactams (24).

Figure 2.10 The location of *ftsI* gene in *H. influenzae* Rd KW20

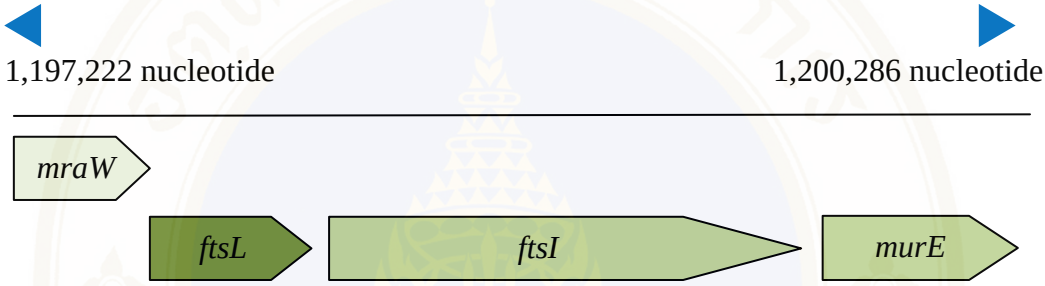

Table 2.13 Genes and gene products for resistance mechanism in *H. influenzae*

Genes	**Gene products**
bla $_{\text{TEM}}$	TEM type beta-lactamase
bla $_{\text{ROB-1}}$	*bla* $_{\text{ROB-1}}$beta-lactamase
ftsI	PBP3
dacA	PBP5
dacB	PBP4
acrA/acrB	AcrAB efflux pump
acrR	AcrR (repressor for AcrAB efflux pump)
tet (B)	Tet(B) tetracycline efflux protein
tet (M), *tet* (K)	Tet(M) and Tet(K) ribosomal protection proteins
cat	Chloramphenicol acetyltransferase

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2001, based on the presence of different amino acid substitutions in transpeptidase region of PBP3, Ubukata et al. can separate BLNAR isolates into 3 groups (I, II, and III). Almost all isolates had D350N and S357N. Group I isolates had R517H. Group II isolates had N526K. Group III isolates had N526K, M377I, S385T, and L389F (179).

2.2.5 Epidemiology of BLNAR

From many studies, BLNAR *H. influenzae* isolates are genotypically diverse with a general absence of clonal spread e.g., in 108 BLNAR isolates tested 70 different pulsed-field gel electrophoresis (PFGE) profiles were found (22, 181). *H. influenzae* has efficient system for uptake homologous DNA, therefore mutated *ftsI* gene may be transferred from BLNAR isolates to susceptible isolates (177).

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Essential oils

Essential oils are aromatic oily liquids obtained from flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots of plant. They can be obtained by fermentation or extraction but the method of steam distillation is the most commonly used for commercial production (182). They are usually used in the production of soaps, perfumes and toiletries (183). Interestingly, essential oils also have antibacterial properties (184) which their antimicrobial properties depend on constituents e.g., structures and possible synergistic interactions between these components (185).

The essential oils used in this study are kaffir lime leaf oil and kaffir lime oil. Kaffir lime from *Citrus hystrix* is a Thai herb. In general, kaffir lime leaf oil contains citronellal (70%) while kaffir lime oil contains beta-pinene (30-40%), limonene (24-30%).

1. History of essential oils

The term ‘essential oil’ comes from the name used in the 16th century by the Swiss reformer of medicine, Paracelsus von Hohenheim who named the effective component of a drug Quinta essential. For a long time, essential oils were recognized that some of them have antimicrobial properties (184). Besides antibacterial properties (186-188), essential oils and their components also exhibit antiviral (189), antimycotic (190, 191), antitoxigenic (184-186), antiparasitic (187, 188), and insecticidal (189, 190) properties.

The first experiment to measure bactericidal property of essential oils was performed by De la Croix in 1881 (185). Antibacterial effect of kaffir lime leaf oil and kaffir lime oil, was reported for greater activity against 20 serotypes of *Salmonella* and 5 species in other enterobacteria, compared to other spice oils (29). Kaffir lime leaf oil showed high antibacterial activity against *Staphylococcus aureus*, while moderate activity against *Salmonella typhi* (27). Kaffir lime leaf, and other oil tested also has strongest antibacterial activity against 5 isolates of *Propionibacterium acnes* (30). Moreover, kaffir lime oil also showed antibacterial activity against extended-spectrum beta-lactamase producing *Klebsiella pneumoniae* (31).

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2. Antibacterial action of essential oils

An important characteristic of essential oil and their components is the hydrophobicity property. A large number of chemical compounds in essential oils, suggested that their antibacterial activity is not caused by one specific mechanism but invoved in several targets in bacterial cell (191, 192). The hydrophobicity property of essential oils cause them to partition in lipids of bacterial cell membrane rupture the structures and cause leakage of cytoplasmic constituents, metabolites and ions; leading to cell death (191, 193-199) (Fig. 2.11) In addition, components in essential oils may act on cell proteins embedded in the cytoplasmic membrane (200).

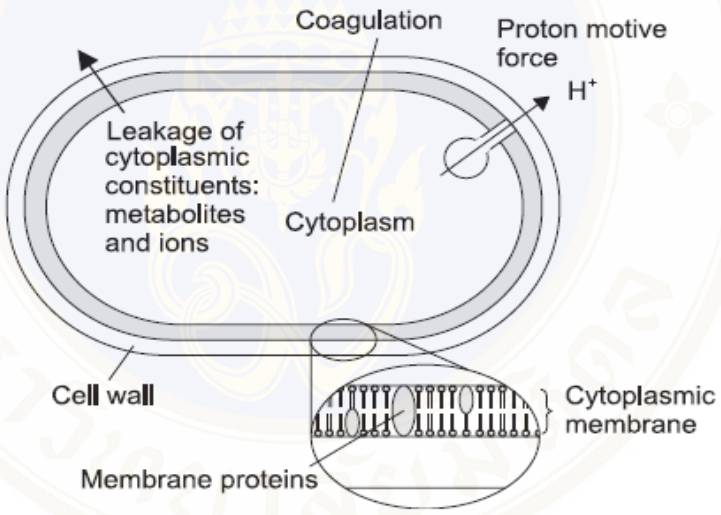

Figure 2.11 Locations and mechanisms in bacterial cell thought to be sites of action for essential oil components

3. Test for antibacterial activity

The antibacterial susceptibility testing according to CLSI is modified for testing essential oils (201). However, there are many factors e.g., method used for extraction of essential oils from plant material, the volume of inoculum, growth phase, culture medium used, pH of media, incubation time and temperature (202) resulting in

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complication in data comparison (203, 204). The techniques used to test antibacterial activity of essential oils are shown in Table 2.14.

Table 2.14 Methods to measure antibacterial activity of essential oils

Purpose of study	**Methods**	
Screening for antibacterial activity	Disk diffusion	
	Agar wells	
Determination of strength of antibacterial properties	Agar dilution method	
	Broth dilution	Visible growth
		Optical density/turbidity
		Absorbance
		Colorimetric
		Conductance/conductivity/impedance
		Viable count
Determination of rapidity and duration of antibacterial activity	Time-kill analysis/ Survival curves	
Observation of physical effects of antibacterial activity	Scanning electron microscopy	

4. Components in essential oils

Essential oils consist of >60 individual components (205, 206). The phenolic components are mainly responsible for antibacterial properties (207) which are the major components of various essential oils with antibacterial properties (Table

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2.15) However, minor components may be a critical part for antibacterial activity by synergistic effect with other components.

Table 2.15 Major components of some essential oils which exhibit antibacterial properties

Common name	**Latin name of plant source**	**Major components**	**Approximate % composition**
Cilantro	*Coriandrum sativum* (immature leaves)	Linalool	26%
		E-2-decanal	20%
Coriander	*Coriandrum sativum* (seeds)	Linalool	70%
		E-2-decanal	–
Oregano	*Origanum vulgare*	Trans-cinnamaldehyde	65%
		Carvacrol	Trace-80%
		Thymol	Trace-64%
		gamma-Terpinene	2 –52%
		p-Cymene	Trace-52%
Rosemary	*Rosmarinus officinalis*	α-Pinene	2 –25%
		Bornyl acetate	0 –17%
		Camphor	2 –14%
		1,8-Cineole	3 –89%
Sage	*Salvia officinalis* L.	Camphor	6 –15%
		α-Pinene	4 – 5%
		β-Pinene	2 –10%
		1,8-Cineole	6 –14%
		α-Tujone	20–42%
Clove (bud)	*Syzygium aromaticum*	Eugenol	75–85%
		Eugenyl acetate	8 –15%
Thyme	*Thymus vulgaris*	Thymol	10–64%
		Carvacrol	2 – 11%
		γ -Terpinene	2 –31%
		p-Cymene	10–56%

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CHAPTER III
MATERIALS AND METHODS

1. Antimicrobial susceptibility by disk diffusion method

1.1 Bacterial isolates

One hundred of beta-lactamase producing *H. influenzae* isolates from various clinical specimens (Table 3.16) were obtained from different patients admitted to Siriraj Hospital during January 2009 to December 2010. Thirty-four of these isolates collected from 2010 and sixty-six isolates collected from 2010 according to consecutive sample collection.

Twenty-six of BLNAR isolates from various clinical specimens (Table 3.16) were obtained from different patients admitted to Siriraj Hospital during January 2008 to December 2010. These BLNAR isolates were collected from all isolates which was beta-lactamase negative and contained ampicillin resistance. Dduring this time, BLNAR group were 7 isolates in 2008, 6 isolates in 2009 and 13 isolates in 2010.

One hundred and twenty-six isolates were identified and performed antimicrobial susceptibility test by using standard microbiological techniques.

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Table 3.16 Sources of specimens

January-December 2008-2010

Clinical specimens	No. of isolates	%
Sputum	70	55.56
Nasal discharge	5	4.03
Ear discharge	1	0.81
Eye discharge	10	8.06
Urethral discharge	1	0.81
Pus	9	7.14
Vaginal swabs	2	1.61
Cervical swabs	2	1.61
Bronchial wash	4	3.23
Tissue biopsy	4	3.23
Bronchoalveolar lavage	12	9.68
Blood	6	4.84
Total	**126**	**100**

1.2 Identification of *H. influenzae*

1.2.1 Cultivation

All isolates were kept at -80˚C and were subcultured on chocolate agar and incubated overnight at 35 °C in 5% CO_2 for 18-24 hrs. They were identified as *H. influenzae* according to standard microbiological techniques (8). Isolates were kept at -80ºC in 5% trypticase soy broth plus 20% v/v glycerol until use. The typical colonial morphology on chocolate agar is flat, grayish-brown colonies with diameters of 1-2 mm.

1.2.2 Chemical and equipments

Antimicrobial susceptibility test was performed using medium and equipments e.g., *Haemophilus* Test Medium (HTM) according to CLSI

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recommendation (8), sterile cotton swab, test tubes, McFarland 0.5 standard tube and nephelometer.

Antimicrobial disks (BD Diagnostics, USA) were ampicillin (10 μg), amoxicillin/clavulanic acid (20/10 μg), cefotaxime (30 μg), ceftriaxone (30 μg), ceftazidime (30 μg), cefuroxime (30 μg), ciprofloxacin (5 μg), tetracycline (30 μg), and trimethoprim-sulphamethoxazole (1.25/23.75 μg).

1.3 Antimicrobial susceptibility test

Disk diffusion method was used as described by CLSI, 2010 (8).

1.3.1 Inoculum preparation

Direct colony suspension method was used for inoculum preparation. The colonies were isolated on chocolate agar. Four to five well isolated colonies were picked from a fresh 16-18 hrs pure culture by using a loop, and emulsified in *Haemophilus* Test Medium (HTM). The turbidity of suspension was adjusted to McFarland 0.5 standard (or 1.0 for mucoid) isolates by using a nephelometer.

1.3.2 Plate inoculation

Within 15 min after standardizing the inoculum, sterile cotton swab was dipped into the suspension, squeezed out excess fluid against the sides of the tube, and swabbed the entire surface of HTM plate in three separate directions (turn 60^{o} after each pass) to achieve smooth, confluent growth on the plate surface.

1.3.3 Apply antimicrobial disks

Within 15 min, antimicrobial disks were applied with slight pressure to ensure that they were not detached from agar surface. The disks were applied to agar surface according to the template provided; with no more than 5 disks per plate. Disks were not removed once they touched agar surface.

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1.3.4 Incubation

The plates were incubated at 35°C in 5% CO_2, 16-18 hrs.

1.3.5 Measurement of inhibition zones

After 16-18 hrs of incubation, the diameters of inhibition zones were read. If a confluent lawn of growth was not achieved, the test would be repeated. The diameters of inhibition zones were measured to the nearest whole millimeter by use of venier calipers or a ruler. The plates were carefully examined to ensure the bacterial growth.

1.3.6 Interpretation

Table 3.17 shows the interpretation criteria of disk diffusion method according to CLSI, 2010 (8).

Table 3.17 Zone diameter interpretive standards (CLSI, 2010)

Antibacterial agents	**Disk content (µg)**	**Diameter of inhibition zone (mm)**		
		Resistant	Intermediate	Susceptible
Ampicillin	10	≤ 18	19-21	≥ 22
Amoxicillin/clavu-lanic acid	20/10	≤ 19	-	≥ 20
Cefotaxime	30	-	-	≥ 26
Ceftriaxone	30	-	-	≥ 26
Ceftazidime	30	-	-	≥ 26
Cefuroxime	30	≤ 16	17-19	≥ 20
Ciprofloxacin	5	-	-	≥ 21
Tetracycline	30	≤ 25	26-28	≥ 29
Trimethoprim-sulphamethoxazole	1.25/23.75	≤ 10	11-15	≥ 16

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1.3.7 Quality control

H. influenzae ATCC 49247 was used as quality control for each day testing. If MIC was out of acceptable limits, whole batch of isolates will be re-tested.

2. Identification of beta-lactamase producing and BLNAR *H. influenzae* isolates by nitrocefin method and determination of minimal inhibitory concentration (MIC) of ampicillin and amoxicillin/clavulanic acid by broth microdilution method and E-test method

2.1 Nitrocefin method

2.1.1 Principle of test

The chromogenic cephalosporin beta-lactamase test is a qualitative procedure for detecting production of beta-lactamase by bacteria. This rapid method utilizes chromogenic cephalosporin compound (nitrocefin) in which when amide bond in beta-lactam ring is hydrolyzed by beta-lactamase, a chromogenic beta-lactamase substrate undergoes distinctive color change from yellow to red.

2.1.2 Procedure

- Whatman No. 1 filter paper disk is placed over a slide.
- One drop of sterile water is placed on a piece of filter paper over a slide.
- Development of red color within 30 sec indicates the presence of beta-lactamase.

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2.2 Determination of MIC of ampicillin by microbroth dilution method

2.2.1 Principle of test

MIC was determined by broth microdilution method as described by CLSI (8). The HTM were used. Antimicrobial susceptibility was tested in two-fold serial dilutions for ampicillin. Testing and preparation of stock solution was prepared according to CLSI (8).

2.2.2 Preparation of *Haemophilus* Test Medium (HTM)

A fresh hematin stock solution was prepared by dissolving 50 mg of hematin powder in 100 ml of 0.01 mol/L NaOH with heat and stirring until the powder was thoroughly dissolved. Then 30 ml of hematin stock was added to 1 L of Mueller-Hinton agar with 5 gm of yeast extract also added. After autoclaving and cooling, 3 ml of an NAD stock solution (50 mg of NAD dissolved in 10 ml of distilled water, filter sterilized) was added aseptically.

2.2.3 Preparation of antimicrobial stock solution

Ampicillin was obtained as standard powder. The latter was accurately weighed and dissolved in appropriate solvent (phosphate buffer pH 8) to yield the required concentration, using sterile glassware and the stock solution can be frozen at -20 ºC or -80 ºC. Stock solution is diluted with diluents (HTM) to yield the required concentration according to CLSI (8).

Stock solution can be prepared using the formula:

$$W = \frac{1000}{P} \times V \times C$$

P = Potency given by the manufacturer in relation to the base

V = Volume in ml required

C = Final concentration of solution

W = Weight of antimicrobial agent to be dissolved in volume V

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2.2.4 Inoculum preparation

Inoculum of *H. influenza*e was prepared from colonies grown on chocolate agar which was incubated overnight at 35 ◦C in 5% CO_2 for 16-18 hrs. Colonies were suspended in HTM and adjusted to turbidity of a 0.5 McFarland standard ($1x10^8$ CFU/ml) and the suspension was further diluted 1:10 with HTM ($1x10^7$ CFU/ml) within 15 min.

2.2.5 Inoculating broth in microtiter plate

Each well of microtiter plate, contained 100 µl sample of HTM with appropriate amount of antimicrobial agent. The inoculum 5 µl volume of bacterial suspension was added into each well and incubated at 35°C in 5% CO_2 for 20-24 hrs before visual determination of MIC. A final inoculum concentration of $5x10^5$ CFU/ml was used.

2.2.6 Determining of MIC end point

The MIC was expressed as the lowest concentration of antimicrobial agent, which completely inhibits growth of *H. influenza*e by lack of turbidity in well(s) of microtiter plate. *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 of known MIC values run with the test were used as quality control to check the reagents and conditions. The amount of growth in wells containing antimicrobial agent was compared with growth in control well (no antimicrobial agent) when determining the growth end point.

2.2.7 Interpretation

The interpretation criteria of ampicillin MIC results are according to CLSI, 2010 (8) (Table 3.18).

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Table 3.18 MIC interpretation in *H. influenzae*

Antibacterial agent	MIC (µg/ml) interpretation		
	Susceptible	Intermediate	Resistant
Ampicillin	≤1	2	≥4

2.2.8 Quality control

Positive control, 5 µl of inoculum of bacterial suspension was added into 100 µl of HTM broth in each well of microtiter plate.

Negative control, only 100 µl of HTM broth was added in each well of microtiter plate.

2.3 Determination of MIC-amoxicillin/clavulanic acid by E-test method for BLNAR *H. influenzae* isolates

2.3.1 Principle of test

E-test is used to determine whether or not a bacterium is susceptible to the action of a specific antibiotic. It is basically an agar diffusion method which utilizes a rectangular strip that has been impregnated with the drug to be studied. A lawn of bacteria is inoculated onto surface of agar and E-test strip is laid on top. After incubation, an elliptical zone of inhibition is produced and the point at which the ellipse meets the strip gives a reading for the MIC of drug (Fig. 3.12).

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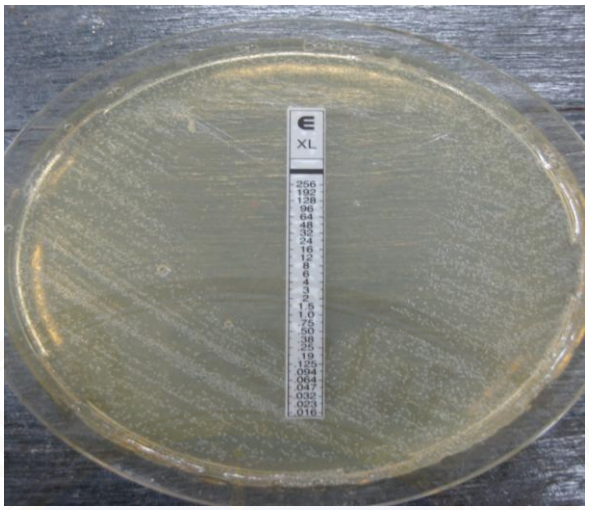

Figure 3.12 Determination of amoxicillin-clavulanic acid MIC by E-test method

2.3.2 Inoculum preparation

The direct colony suspension method as previously described was used for inoculum preparation.

2.3.3 Plate inoculation

Within 15 min after standardizing the inoculum, sterile cotton swab was dipped into the suspension, squeezed out excess fluid against the sides of the tube, and swabbed the entire surface of the *Haemophilus* Test Medium plate in three separate directions (turn 60^{o} after each pass) to achieve smooth, confluent growth on the plate surface.

2.3.4 Apply E-test strips

Within 15 min, E-test strip of amoxicillin/clavulanic acid (AB BIODISK, Sweden) were applied with forceps. Strips were not removed once they touched the agar surface.

2.3.5 Incubation

The plates were incubated at 35^{o}C in 5% CO_2 for 20-24 hrs.

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2.3.6 Measurement of inhibition zones and reading MIC

After 20-24 hrs of incubation, the MIC was read. If a confluent lawn of growth was not achieved, the test would be repeated. The MIC is determined as the point where the elliptical zone of inhibition intersects the E-test strip edge. The end point is the limit of the area showing no obvious growth visible to the naked eyes.

2.3.7 Interpretation

The interpretation criteria of amoxicillin/clavulanic acid MIC results are according to CLSI (8). MIC ≥ 8 µg/ml is considered resistant (8).

2.3.8 Quality control

H. influenzae ATCC 49247 is used as quality control for each day testing. If MIC is out of acceptable limits, whole batch of isolates will be re-tested.

3. Molecular technique for detection of *bla*$_{TEM}$ and *bla*$_{ROB-1}$ genes in beta-lactamase producing *H. influenzae* isolates and *ftsI* genes in BLNAR *H. influenzae*

One-hundred out of one-hundred and twenty-six clinical isolates from respiratory tract infection, which were resistant to ampicillin (MIC ≥4 µg/ml) were tested for the presence of *bla*$_{TEM}$ and *bla* $_{ROB-1}$ genes. Twenty-six isolates, which were also resistant to ampicillin were tested for the presence of *ftsI* gene by polymerase chain reaction (PCR) method as follows:

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3.1 Primer design (Table 3.19)

Table 3.19 Nucleotide sequences of primers for detections of bla_{TEM}, bla_{ROB-1}, *ftsI* genes and PCR product length

Primers	**Nucleotide sequences (5´→3´)**	**Product length**	**Reference**
bla_{TEM} F	ATGAGTATTCAACATTTCCG	863 bp	(208)
bla_{TEM} R	CTGACAGTTACCAATGCTTA		
bla_{ROB-1} F	AAGCCCGCATTGGCGTTTAT	231 bp	(16)
bla_{ROB-1} R	GGGCAATCGTCATGCCTTTG		
ftsI F	GTGTTAGCTATGGCGACTGC	875 bp	This study
ftsI R	TCTTGCGGAATAGCATTTG		
ftsI walking F	CGTAAACGCTGGGCAGATA	104 bp	This study
ftsI walking R	AAACTACCAAGGGTTGCAT		

The *ftsI* primers were designed from *ftsI* gene between 796 and 1,741 bp, which encoded transpeptidase region of PBP 3, found in all *H. influenzae* isolates from NCBI database. The *ftsI fo*rward primer which was designed in this study was located in conserved region at 874 bp and 1,748 bp for *ftsI* forward and reverse primers (Fig. 3.13).

The *ftsI* walking primers in this study were designed outward from nucleotide sequence obtained from the first round sequencing which was located in conserved region at 1,273 bp and 1,376 bp for *ftsI* walking forward and reverse primers (Fig. 3.14).

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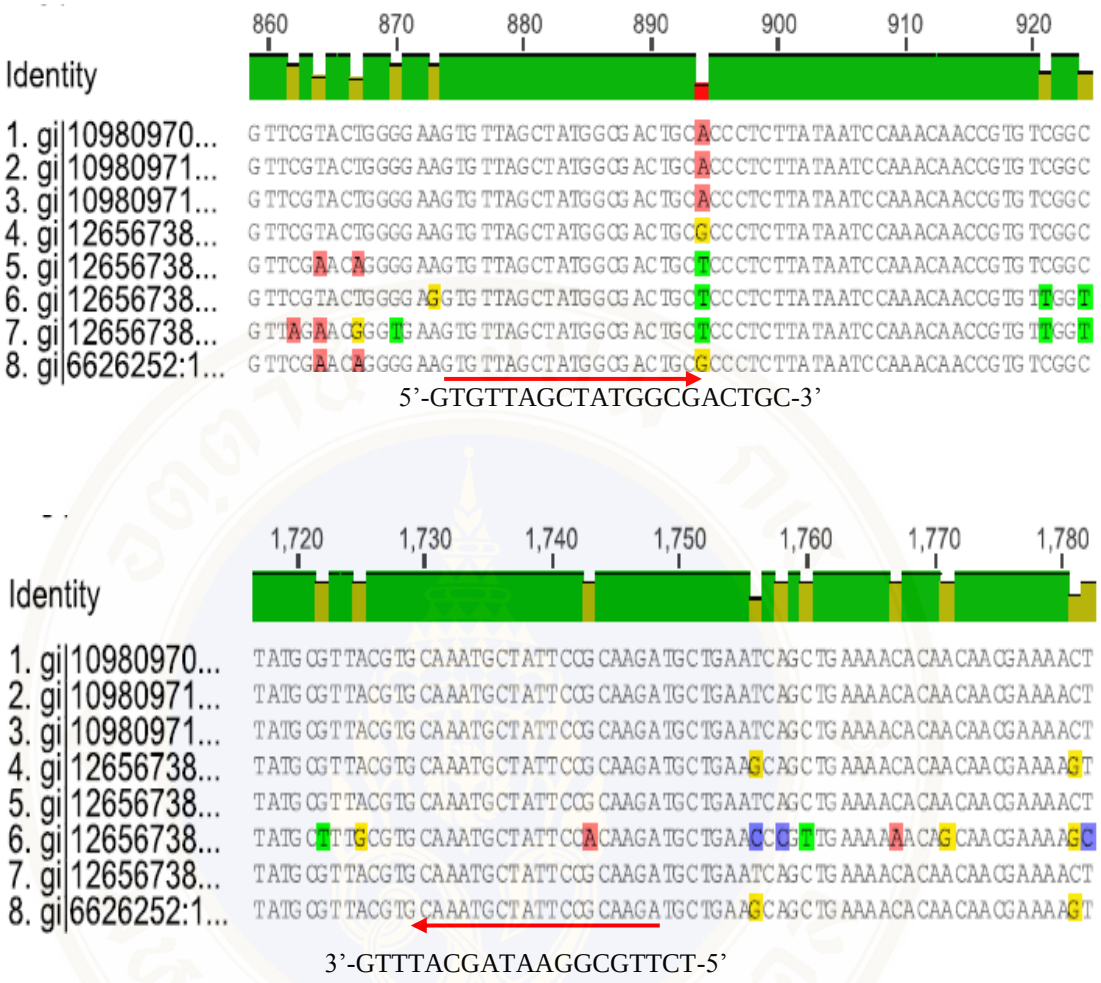

Figure 3.13 The *ftsI* forward and reverse primers which are indicated by red arrows

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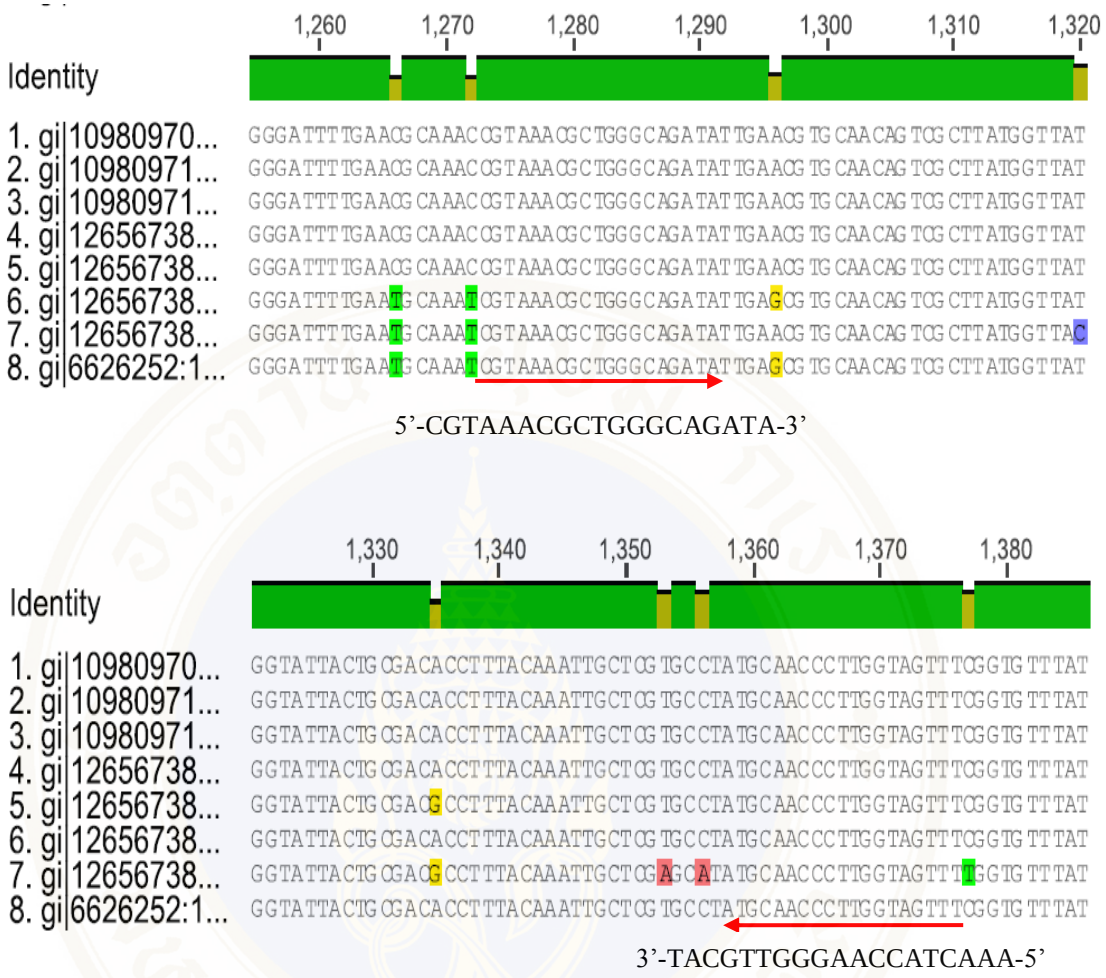

Figure 3.14 The *ftsI* walking forward and reverse primers which are indicated by red arrows

3.2 DNA extraction

Total genomic DNA from 126 isolates was extracted by using a Puregene DNA purification kit (Gentra Systems, USA).

A loopful of colonies of each isolate taken from 16-18 hrs culture on chocolate agar at 35°C in 5% CO_2 incubator was suspended in 300 µl of cell suspension solution in a 1.5 ml microcentrifuge tube and gently pipetted up and down until cells were suspended. 1.5 µl of lytic enzyme solution was added into the suspension and mixed gently by inverting the tube 25 times. The suspension was incubated at room temperature for 30 min to digest cell wall occasionally inverting it during the incubation. After incubation, the suspension was centrifuged at 13,000 x g

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for 1 min to pellet bacterial cells, and then supernatant was removed. 300 µl of cell lysis solution was added to the cell pellet and gently pipetted up and down to lyse the cells. 1.5 µl RNase A solution was added to the cell lysate and mixed gently by inverting the tube 25 times. The suspension was incubated at room temperature for 30 min. After that, sample was cooled to room temperature by placing on ice for 1 min. Later, 100 µl protein precipitation solution was added to cell lysate and vortexed vigorously at high speed for 20 sec to mix the protein precipitation solution uniformly with the cell lysate. The cell lysate was centrifuged at 13,000 x g for 3 min to obtain a tight white pellet. The supernatant containing DNA was poured into a clean 1.5 ml microcentrifuge tube, which already contained 300 µl 100% isopropanol (2-propanol). The sample was mixed gently by inverting the tube 50 times. It was then centrifuged at 13,000 x g for 1 min. The DNA was visible as a small white pellet. The supernatant was poured off and the tube was drained briefly on clean absorbent paper. Three hundred µl of 70% ethanol was added and the tube was inverted several times to wash the DNA pellet. The supernatant was centrifuged at 13,000 x g for 1 min. The ethanol was carefully poured off. The tubes was inverted, drained on clean absorbent paper and allowed to air dry 30 min. 100 µl of DNA hydration solution was added. DNA was rehydrated by incubating sample for 1 hr at 65ºC and overnight at room temperature. The tube was tapped periodically to aid in dispersing DNA. The genomic DNA was stored at 4ºC. For long term storage, each sample was placed at -20ºC or -80ºC.

3.3 PCR amplification of *bla*$_{\text{TEM}}$ and *bla*$_{\text{ROB-1}}$ genes in beta-lactamase producing and *ftsI* genes in BLNAR *H. influenzae* isolates

The *bla*$_{\text{TEM}}$, *bla*$_{\text{ROB-1}}$ and *ftsI* genes were detected by the PCR amplification from genomic DNA.

The PCR amplification of *bla*$_{\text{TEM}}$, *bla*$_{\text{ROB-1}}$ and *ftsI* genes was performed in a 25 µl polymerase chain reaction mixture i.e., 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.5 µM each primer, 200 µM each deoxynucleoside triphosphate and 1 U of *Taq* DNA polymerase (DyNAzyme^{TM} II DNA Polymerase, Roche Co. Ltd., USA). The concentration and volume of each reagent in polymerase chain reaction mixture was shown in Table 3.20. The PCR cycling was performed in the PERKIN

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ELMER GeneAmp PCR System 2400 by using the following conditions: pre-denaturation at 94°C for 5 min; 30 cycles of 30 sec at 94 °C, 30 sec at 55 °C and 1 min at 72 °C; and final extension at 72 °C for 7 min for *bla* $_{TEM}$ gene, pre-denaturation at 94°C for 5 min; 30 cycles of 15 sec at 94 °C, 15 sec at 54.2 °C and 15 sec at 72 °C; and final extension at 72 °C for 7 min for *bla* $_{ROB-1}$ gene and 94°C for 5 min; 30 cycles of 30 sec at 94 °C, 30 sec at 55 °C and 1 min at 72 °C; and final extension at 72 °C for 7 min for *ftsI* gene. The amplified products were detected by electrophoresis in a 1% agarose gel (Ultra pure agarose electrophoresis grade, Life Technologies Inc., USA) containing 1X Tris-acetate-EDTA buffer. Prior to electrophoresis, samples were mixed with loading dye. The gel was run in 1X Tris-acetate-EDTA buffer at 100 volts for 30 min and a 1 Kb plus DNA ladder was used as a size marker. Following electrophoresis, the gel was stained with ethidium bromide and photographed by a gel doc system (Biorad Co. Ltd., USA).

Table 3.20 Reagents in PCR mixture for *bla*$_{TEM}$, *bla*$_{ROB-1}$ and *ftsI* genes

Reagents	**Volume (µl): 1 sample**
10X PCR buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl, 1% Triton X-100 and 15 mM $MgCl_2$]	2.5
Forward primer (100 µM)	0.25
Reverse primer (100 µM)	0.25
10 mM dNTPs	0.5
DNA polymerase (2U/µl)	0.5
Total genomic DNA	1
25 mM $MgCl_2$	2
MilliQ water	18
Total volume	**25**

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3.3.1 PCR amplification for both *bla*$_{TEM}$ and *bla*$_{ROB-1}$ genes in the same beta-lactamase producing *H. influenzae* isolates

All isolates which were only *bla*$_{TEM}$ gene positive or *bla*$_{ROB-1}$ positive were performed PCR amplification in order to detect isolates which were positive for both *bla*$_{TEM}$ and *bla*$_{ROB-1}$ genes.

3.4 Identification type of *bla*$_{TEM}$ gene and detection mutations in target genes of BLNAR *H. influenzae* isolates (*ftsI* genes)

One in one-hundred isolates which were positive in beta-lactamase was identified for a type of *bla*$_{TEM}$ gene. Five in twenty-six isolates which were negative in beta-lactamase and resistant to ampicilln were identified for mutations in *ftsI* gene causing altered PBP3.

3.4.1 Cloning PCR product in vector

PCR product of *bla*$_{TEM}$ and *ftsI* genes was cloned into StrataClone PCR Cloning (Agilent Technologies company, USA).

The StrataClone PCR cloning vector mix contains two DNA arms, each charged with topoisomerase I on one end and containing a *loxP* recognition sequence on the other end. The topoisomerase-charged ends have a modified uridine (U*) overhang. *Taq*-amplified PCR products, which contain 3´-adenosine overhangs, are efficiently ligated to these vector arms in a 5 min ligation reaction, through A-U* base-pairing followed by topoisomerase I-mediated strand ligation.

The insert DNA was prepared by PCR using *Taq* DNA polymerase. The ligation reaction mixture was prepared by combination of 3 µl StrataClone Cloning Buffer, 2 µl of PCR product and 1 µl StrataClone Vector Mix amp/kan. Ligation reaction mixture was incubated at room temperature for 5 min then placed on ice. 1 µl of cloning reaction mixture was added to a tube of thawed StrataClone SoloPack competent cells and mixed gently. The transformation mixture was incubated on ice for 20 min then heat-shocked at 42°C for 45 sec. Transformation mixture was incubated on ice for 2 min. 250 µl of LB medium was added to tube containing transformation mixture to allow the cells to recover at 37°C with agitation for at least 1 hr (incubate for 1.5–2 hrs before plating on kanamycin plates). 100 µl

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transformation mixture was plated on LB–ampicillin or LB-kanamycin plates that have been spread with 40 µl of 2% X-gal. The plates were incubated overnight at 37°C. White or light blue colonies were picked for plasmid DNA analysis. Miniprep DNA was prepared from the selected colonies.

3.4.2 Plasmid extraction

After PCR product was cloned into vector, plasmid extraction was then performed by High-Speed Plasmid Mini Kit (Geneaid Biotech Ltd., Taiwan). This kit was designed for rapid isolation of plasmid DNA from 1-4 ml of cultured bacterial cells. Modified alkaline lysis method and RNase treatment are used to obtain clear cell lysate with minimal genomic DNA and RNA contaminants.

Step 1. Harvesting: 1.5 ml of cultured bacterial cells was transferred to a microcentrifuge tube then centrifuged at 14-16,000 x g for 1 min and discarded the supernatant.

Step 2. Resuspension: 200 µl of PD1 Buffer (RNase A added) was added to the tube and resuspended the cell pellet by vortex or pipetting.

Step 3. Lysis: 200 µl of PD2 Buffer was added and mixed gently by inverting the tube 10 times. Do not vortex to avoid shearing the genomic DNA and let stand at room temperature for 2 min.

Step 4. Neutralization: 300 µl of PD3 Buffer was added and mixed immediately by inverting the tube 10 times. Do not vortex. The mixture was centrifuged at 14-16,000 x g for 3 min.

Step 5. DNA binding: a PD column was placed in a 2 ml collection tube. The supernatant from step 4 to the PD column and centrifuged at 14-16,000 x g for 30 sec. The flow-through was discarded and then placed the PD column back in the 2 ml collection tube.

Step 6. Wash: 400 µl of W1 Buffer was added into the PD column and centrifuge at 14-16,000 x g for 30 sec. The flow-through was discarded and placed the PD column back in the 2 ml collection tube. 600 µl of Wash Buffer (ethanol added) was added into the PD column and centrifuged at 14-16,000 x g for 30 sec. The flow through was discarded and placed the PD column back in the 2 ml

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collection tube. The tube was centrifuged at 14-16,000 x g again for 3 min to dry the column matrix.

Step 7. DNA Elution: The dried PD column was transferred to a new microcentrifuge tube. 50 µl of elution buffer or TE was added into the center of the column matrix. Let stand for 2 min or until the elution buffer or TE was absorbed by the matrix. The tube was centrifuged at 14-16,000 x g for 2 min to elute the DNA.

3.4.3 Restriction enzyme digestion

Plasmids containing the PCR product insert were identified and determined insert orientation by restriction analysis by using *Eco*RI-HF (Fig. 3.15).

The condition for restriction analysis:

Milli Q water	10 µl
*Eco*RI -HF	1 µl
Buffer	2 µl
Plasmid	7 µl
Total	20 µl

Figure 3.15 pSC-A PCR cloning vector PCR product insertion site region

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3.5 DNA sequencing of *bla*$_{TEM}$ and *ftsI* genes

The purified plasmid was verified by DNA sequencing with M13 for *bla*$_{TEM}$ and *ftsI* genes and *ftsI* walking primer for sequence outward from M13 primer in *ftsI* gene (Ward Medic Ltd., Thailand) in order to confirm the sequence of *bla*$_{TEM}$ and *ftsI* genes.

3.6 BLAST sequence with GenBank database and program of bioinformatics tools

DNA sequences of of *bla*$_{TEM}$ and *ftsI* genes were used to perform nucleotide blast (blastn) and protein blast (blastx) in GenBank database from website: http://ncbi.nlm.nih.gov.

3.7 Restriction analysis

This analysis was performed to determine the difference in each sequence of BLNAR isolates. PCR product of each BLNAR isolate was digested with 4-base cutter enzymes which were *Sau*3AI and *Afa*I.

*Sau*3AI is DNA restriction enzyme from *Staphylococcus aureus* 3A which is supplied in 10 mM Tris-HCl, 100 mM KCl, 0.1 mM EDTA, 10 mM 2-mercaptethanol, 0.01% BSA and 50% glycerol (pH 7.5). The recognition site is shown in Fig. 3.16

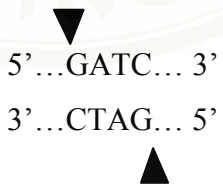

Figure 3.16 The recognition site of *Sau*3AI enzyme

*Afa*I is DNA restriction enzyme from *Acidiphilium facilis* which is supplied in 10 mM Tris-HCl, 100 mM KCl, 0.1 mM EDTA, 10 mM DTT, 0.02% BSA and 50% glycerol (pH 7.5). The recognition site is shown in Fig. 3.17

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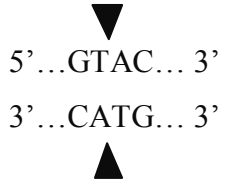

Figure 3.17 The recognition site of *Afa*I enzyme

The condition for *Sau*3AI:

Milli Q water	10 µl
*Sau*3A I	1 µl
Buffer H	2 µl
ftsI PCR product	7 µl
Total	20 µl

The total amount of digested tube was activated at 37 ºC for 1 hr and inactivated at 60 ºC for 15 min.

The condition for *Afa*I:

Milli Q water	8 µl
*Afa*I	1 µl
10 X T Buffer	2 µl
0.1% BSA	2 µl
ftsI PCR product	7 µl
Total	20 µl

The total amount of digested tube was activated at 37 ºC for 1 hr and inactivated at 60 ºC for 15 min. After complete digestion, the result was examined by agarose gel electrophoresis.

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4. Antimicrobial susceptibility test for kaffir lime leaf oil and kaffir lime oil

Disk diffusion and broth microdilution method were performed in *H. influenzae* and other respiratory tract pathogens including Group A, B, C, F, G streptococci, *S. pneumoniae, S. aureus* (MRSA and MSSA) and *A. baumannii*, totally 359 isolates.

4.1 Disk diffusion method

4.1.1 Principle of test

Kaffir lime leaf oil and kaffir lime oil will diffuse from a paper disk into agar medium that contains test organisms. Inhibition is observed as a failure of bacteria to grow in the region around the disk containing kaffir lime leaf oil and kaffir lime oil. This area of no growth around the disk is known as a "zone of inhibition".

4.1.2 Reagents and materials

- Peptone broth
- Kaffir lime leaf oil
- Kaffir lime oil
- Whatman disk diameter 6 mm
- Agar medium
- Cotton swab
- Forceps

4.1.3 Agar medium

Chocolate agar was used for *H. influenzae*, sheep blood agar was used for Group A, B, C, F, G streptococci and *S. pneumoniae*, Muller Hinton agar was used for *S. aureus* (MRSA and MSSA), *A. baumannii*.

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4.1.4 Specimen preparation

Prior to testing, all isolates were incubated overnight at 35 ºC in ambient incubator for 16-18 hrs except Group A, B, C, F, G streptococci, *S. pneumoniae* and *H. influenzae* which were incubated at 35 ◦C in 5% CO_2 for 16-18 hrs.

4.1.5 Procedure

- A sterile loop was used to pick up 4-5 colonies from the agar plate into peptone broth and mix thoroughly making sure turbidity of a 0.5 McFarland standard ($1x10^8$ CFU/ml).

- The swab was dipped into the broth culture of the organism and gently squeezed against the inside of the tube to remove excess fluid.

- The swab was used to streak agar plate in three directions.

- Autopipette was used to pipette 10 µl each of kiffir lime leaf oil and kaffir lime oil on a blank disk

- Flame sterilized forceps were used to place disk containing kiffir lime leaf oil and kaffir lime oil onto the surface of the agar.

- The plates were incubated at 35 ºC in ambient incubator for 18-24 hrs except Group A, B, C, F, G streptococci, *S. pneumoniae*, and *H. influenzae* were incubated at 35ºC in 5% CO_2 for 18-24 hrs.

4.1.6 Quality control

10 µl of DMSO solvent was added to blank disk as a negative control and positive control was ampicillin disk.

4.2 Determination Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) by microbroth dilution method

4.2.1 Principle of test

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This method is used to determine susceptibility of bacteria to kaffir lime leaf oil and kaffir lime oil and is considered quantitative method. The result is interpreted as MIC and MBC.

4.2.2 Reagents and materials

- Peptone broth
- Kaffir lime leaf oil
- Kaffir lime oil
- Tween 20
- 96 well microtiter plate
- Broth medium: *Haemophilus* Test Medium (HTM) for *H. influenzae*, cation–adjusted Mueller Hinton broth (CAMHB) supplemented with 2-5% lysed horse blood (LHB) for Group A, B, C, F, G streptococci and *S. pneumoniae* and cation–adjusted Mueller Hinton broth (CAMHB) for *S. aureus* (MRSA and MSSA) and *A. baumanii*.

4.2.3 Procedure

- Broth plus tween 20 in concentration of 0.25 %v/v was prepared and added into 96 well microtiter plate.
- Kaffir lime leaf oil and kaffir lime oil were diluted two-fold serial dilutions with broth medium in range 0.015-8 %v/v in 96 well microtiter plate containing 100 µl of broth.
- A sterile loop was used to pick up 4-5 colonies from agar plate into peptone broth and mix thoroughly making sure turbidity of a 0.5 McFarland standard ($1x10^8$ CFU/ml) and the suspension was further diluted 1:10 ($1x10^7$ CFU/ml) within 15 min.
- 5 µl of bacterial suspension was inoculated into each well of microtiter plate. A final inoculum concentration of $5x10^5$ CFU/ml was obtained.
- Each bacterial suspension was incubated at 35 ºC in ambient incubator for 18-24 hrs before visual determination of the MIC.
- After determination of the MIC, all clear wells were plated onto agar and incubated at 35 ºC in ambient incubator for *S. aureus* (MRSA and

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MSSA) and *A. baumanii* or in 5% CO_2 for Group A, B, C, F, G streptococci, *S. pneumoniae* and *H. influenzae* for 18-24 hrs to determine the MBC.

4.2.4 Quality control

100 µl of broth and 5 µl of bacterial suspension were used as positive control and only 100 µl of broth was used as negative control.

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CHAPTER IV
RESULTS

Beta-lactamase producing *H. influenzae* isolates

1. Antimicrobial susceptibility

For 100 beta-lactamase producing *H. influenzae* isolates, the ratio of male: female patients was 1.26: 1. The age range was 2 months-98 years. The patient age groups were: <2 years (9%), 2-9 years (11%), 10-19 years (4%), 20-29 years (8%), 30-39 years (6%), 40-49 years (12%), 50-59 years (11%), ≥ 60 years (35%) and uncompleted data (4%); most isolates were recovered from adults. These isolates tested to 9 antimicrobial agents by disk diffusion method, they were susceptible to cefotaxime, ceftriaxone, ciprofloxacin (100% each), followed by cefuroxime (96%), amoxicillin/clavulanic acid (88%), ceftazidime (67%), tetracycline (47%), and trimethoprim-sulphamethoxazole (28%). None of the isolates tested was susceptible to ampicillin (Table 4.21 and Fig. 4.18)

2. Correlation of beta-lactamase production and the susceptibility to beta-lactams

Table 4.22 shows correlation between beta-lactamase production and the susceptibility to beta-lactams i.e., ampicillin, amoxicillin/clavulanic acid, cefotaxime, ceftriaxone, ceftazidime and cefuroxime. 100 beta-lactamase producing isolates showed 100% resistance to ampicillin but other beta-lactams e.g., cefotaxime and ceftriaxone still 100% susceptible, followed by cefuroxime (96%), ceftazidime (67%) and amoxicillin/clavulanic acid (88%).

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3. Determination of minimal inhibitory concentrations in beta-lactamase producing isolates

The results of 100 *H. influenzae* isolates which were positive for beta-lactamase production by nitrocefin method and ampicilln-MIC values by broth microdilution method are shown in Table 4.23 and Fig. 4.19

4. Detection of types of beta-lactamase genes

100 beta-lactamase producing *H. influenzae* isolates were detected for bla_{TEM} and bla_{ROB-1} genes by PCR technique. The 863 bp of bla_{TEM} gene and 231 bp of bla_{ROB-1} gene fragments were amplified (Figs 4.20 and 4.21). The ratio of types of beta-lactamase enzyme detected by PCR technique revealed bla_{TEM} type was predominated (99%) (Fig. 4.22).

After gel electrophoresis, PCR product of one randomly selected of bla_{TEM} positive was cloned into StrataClone™ PCR cloning vector pSC-A. Then plasmids containing the PCR product insert were identified and determined insert orientation by restriction analysis by using *Eco*RI (Fig. 4.23). A DNA sequence of this bla_{TEM} positive was analyzed by software available over the internet at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignment of sequence was analyzed by Bioedit program.

The DNA sequence analysis revealed a coding region of 286 amino acids (Fig. 4.24). The result was 100% nucleotide and amino acid sequences identity with the published sequence of bla_{TEM-1} gene in some bacteria e.g., *Enterobacter aerogenes* plasmid pYS10 beta-lactamase.

The amino acids found in the bla_{TEM} gene were Gln6, Phe16, Leu21, Glu28, Lys34, Asp35, Asp38, Gln39, Leu40, Ala42, Arg43, Val44, Leu49, Leu51, Glu64, Met69, Val80, Val84, Gly92, Asn100, Leu102, Glu104, Thr114, Asp115, Arg120, Ser124, Ile127, Ser130, Pro145, Lys146, His153, Met155, Asp157, His158, Asp163, Arg164, Try165, Ile173, Asn175, Arg178, Asp179, Met182, Ala184, Thr189, Gly196, Arg204, Glu212, Ala213, Gly218, Leu221, Ala224, Phe230, Ala237, Gly238, Glu240, Arg244, Ala249, Val262, Thr265, Ser268, Arg275, Asn276, Ile279, Ala280,

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Gly283, Ala284 and His289, respectively which correlated with TEM-1 beta-lactamase (Fig. 4.25).

Table 4.21 Antimicrobial susceptibility of beta-lactamase producing *H. influenzae* by disk diffusion method for 100 isolates

Antimicrobial agents	**Number (%) of susceptibility**		
	Susceptible	Intermediate	Resistant
Ampicillin	-	-	100
Amoxicillin/clavulanate acid	88	-	12
Cefotaxime	100	-	-
Ceftriaxone	100	-	-
Ceftazidime	67	-	33
Cefuroxime	96	1	3
Ciprofloxacin	100	-	-
Tetracycline	47	10	43
Trimethoprim-sulphamethoxazole	28	-	72

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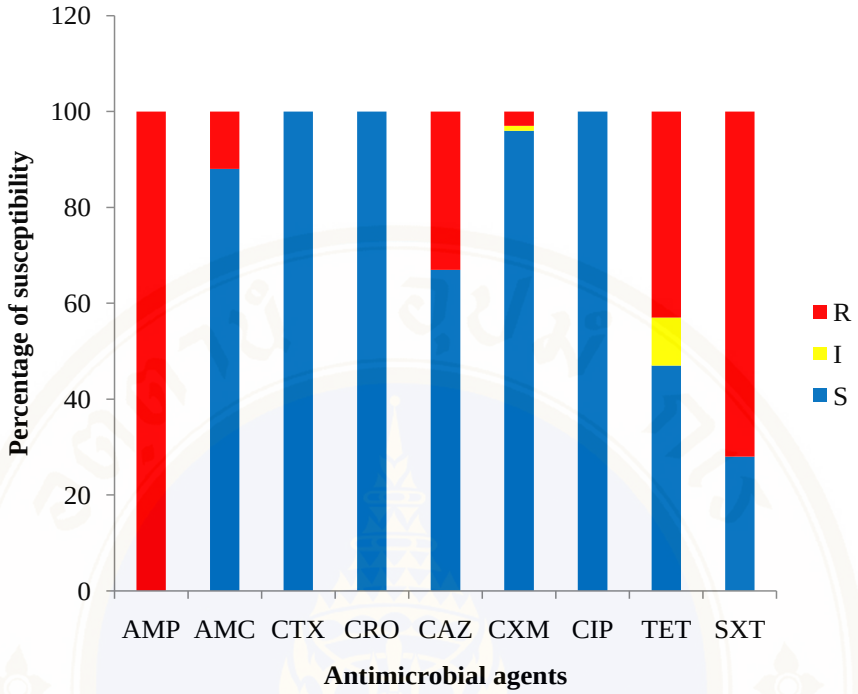

Figure 4.18 Antimicrobial susceptibility of beta-lactamase producing *H. influenzae*

Abbreviation: AMP = ampicillin, AMC = amoxicillin/clavulanic acid,
CTX = cefotaxime, CRO = ceftriaxone, CAZ = ceftazidime, CXM = cefuroxime,
CIP = ciprofloxacin, TET = tetracycline and SXT = trimethoprim-sulphamethoxazole.
S = susceptible, I = intermediate, R = resistant.

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Table 4.22 Correlation of beta-lactamase production and beta-lactams susceptibility in 100 beta-lactamase producing isolates

Beta-lactams	**S (%)**	**I (%)**	**R (%)**
AMP	-	-	100
AMC	88	-	12
CTX	100	-	-
CRO	100	-	-
CAZ	67	-	33
CXM	96	1	3

Abbreviation: AMP = ampicillin, AMC = amoxicillin/clavulanic acid,
CTX = cefotaxime, CRO = ceftriaxone, CAZ = ceftazidime, CXM = cefuroxime,
CIP = ciprofloxacin, TET = tetracycline and SXT = trimethoprim-sulphamethoxazole.
S = susceptible, I = intermediate, R = resistant.

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Table 4.23 Minimal inhibitory concentrations of ampicillin in beta-lactamase producing *H. influenzae*

	Minimal inhibitory concentrations (µg/ml)							
	8	16	32	64	128	256	512	>512
No. of isolates	4	4	6	5	25	17	17	22

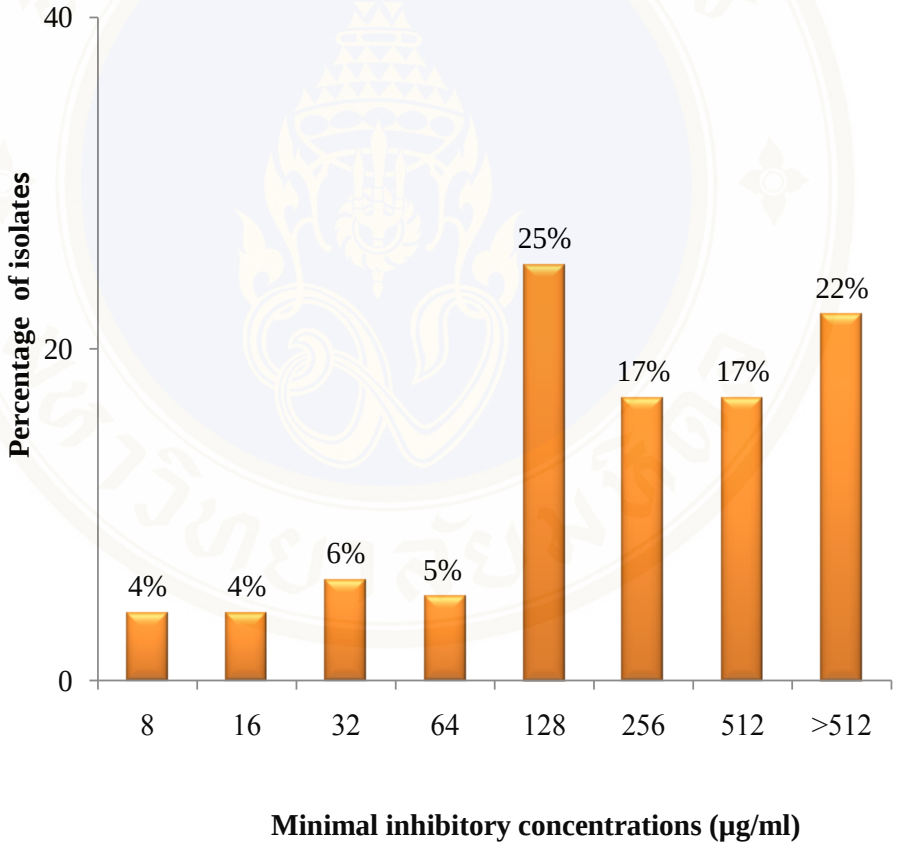

Figure 4.19 Minimal inhibitory concentrations of ampicillin in beta–lactamase producing *H. influenzae*

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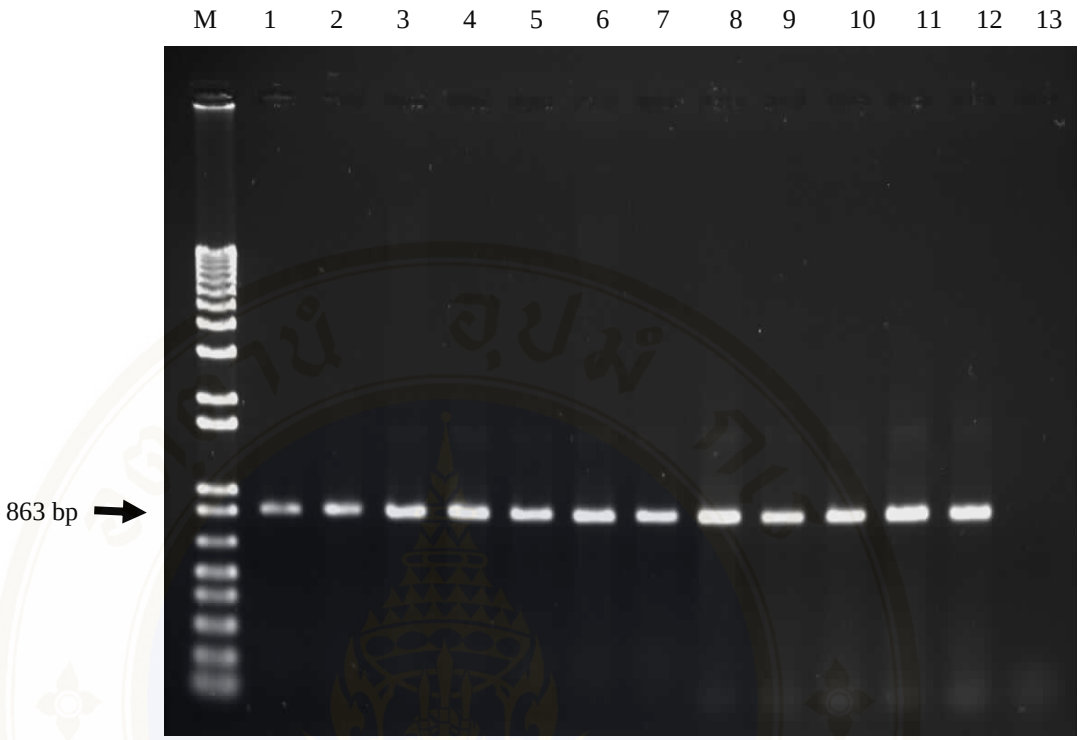

Figure 4.20 PCR products of 863 bp bla_{TEM} gene fragment of *H. influenzae*
Lane M was the standard 1 kb plus DNA size marker.
Lanes 1-11 were bla_{TEM} gene carrying 863 bp fragment of bla_{TEM} positive. Lane 12 was bla_{TEM} gene carrying 863 bp fragment of *N. gonorrhoeae* (positive control). Lane 13 was negative control.

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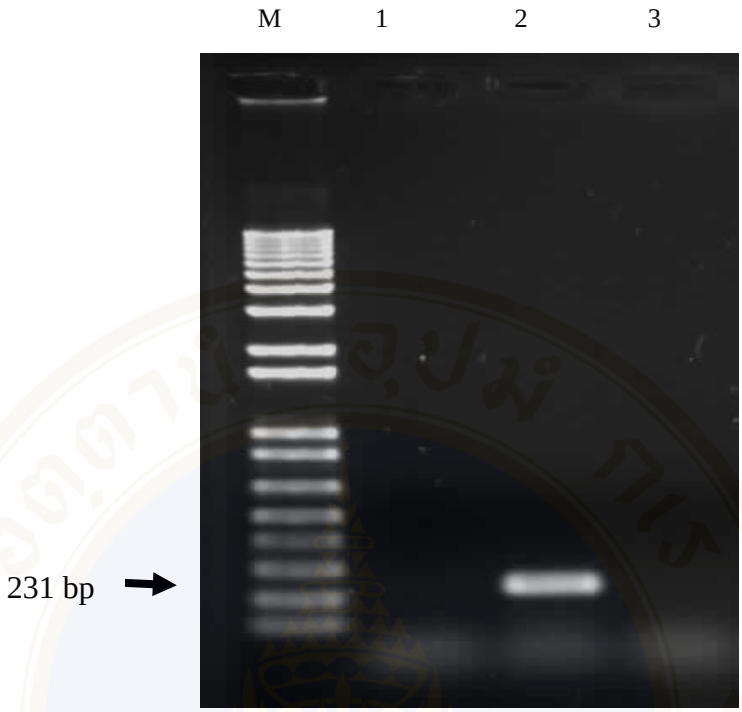

Figure 4.21 PCR product of 231 bp bla_{ROB-1} gene fragment of *H. influenzae*
Lane M was the standard 1 kb plus DNA size marker.
Lanes 1 was isolate without bla_{ROB-1} gene. Lane 2 was bla_{ROB-1} gene carrying 231 bp fragment of *H. influenzae* isolates. Lane 3 was negative control.

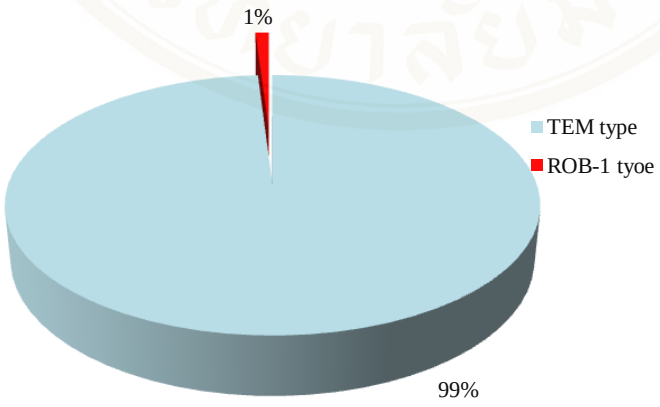

Figure 4.22 Types of beta-lactamase

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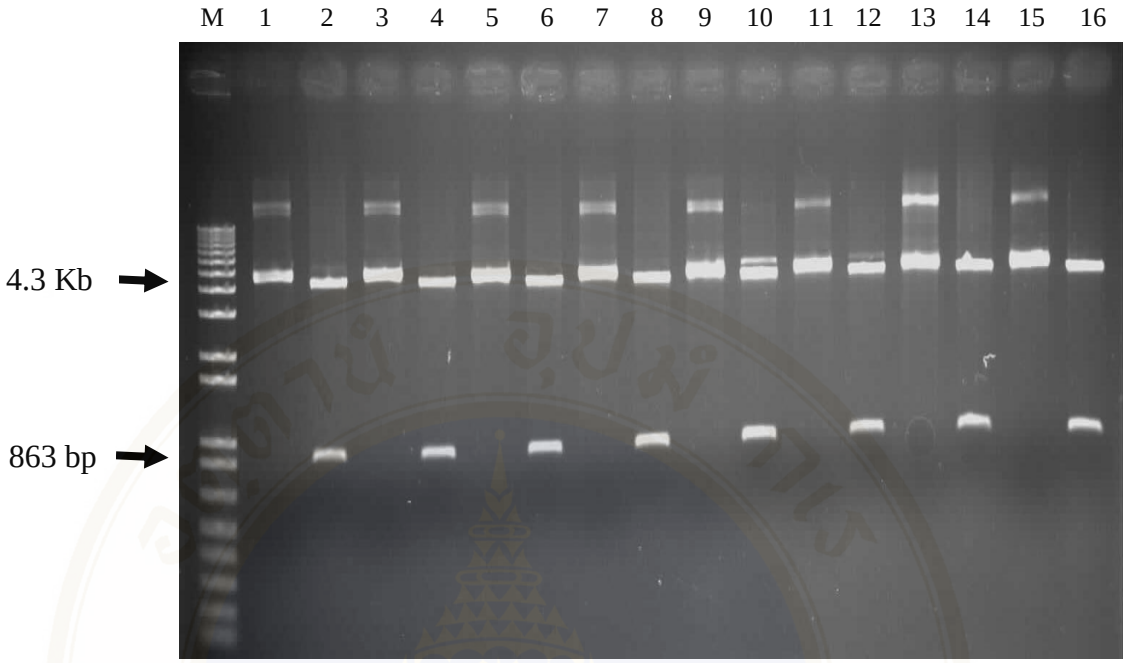

Figure 4.23 Insertion of plasmids containing PCR product (bla_{TEM}) by *Eco*RI restriction analysis

Lane M was the standard 1 kb plus DNA size marker. Lanes 1, 3, 5, 7, 9, 11, 13, 15 = undigested plasmids containing PCR product of bla_{TEM} insertion. Lanes 2, 4, 6, 8, 10, 12, 14, 16 = digested plasmids containing PCR product of bla_{TEM} insertion.

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```
1   ATGAGTATTC AACATTTCCG TGTCGCCCTT ATTCCCTTTT TTGCGGCATT TTGCCTTCCT
    M  S  I  Q  H  F  R  V  A  L  I  P  F  F  A  A  F  C  L  P

61  GTTTTTGCTC ACCCAGAAAC GCTGGTGAAA GTAAAAGATG CTGAAGATCA GTTGGGTGCA
    V  F  A  H  P  E  T  L  V  K  V  K  D  A  E  D  Q  L  G  A

121 CGAGTGGGTT ACATCGAACT GGATCTCAAC AGCGGTAAGA TCCTTGAGAG TTTTCGCCCC
    R  V  G  Y  I  E  L  D  L  N  S  G  K  I  L  E  S  F  R  P

181 GAAGAACGTT TTCCAATGAT GAGCACTTTT AAAGTTCTGC TATGTGGTGC GGTATTATCC
    E  E  R  F  P  M  M  S  T  F  K  V  L  L  C  G  A  V  L  S

241 CGTGTTGACG CCGGGCAAGA GCAACTCGGT CGCCGCATAC ACTATTCTCA GAATGACTTG
    R  V  D  A  G  Q  E  Q  L  G  R  R  I  H  Y  S  Q  N  D  L

301 GTTGAGTACT CACCAGTCAC AGAAAAGCAT CTTACGGATG GCATGACAGT AAGAGAATTA
    V  E  Y  S  P  V  T  E  K  H  L  T  D  G  M  T  V  R  E  L

361 TGCAGTGCTG CCATAACCAT GAGTGATAAC ACTGCTGCCA ACTTACTTCT GACAACGATC
    C  S  A  A  I  T  M  S  D  N  T  A  A  N  L  L  L  T  T  I

421 GGAGGACCGA AGGAGCTAAC CGCTTTTTTG CACAACATGG GGGATCATGT AACTCGCCTT
    G  G  P  K  E  L  T  A  F  L  H  N  M  G  D  H  V  T  R  L

481 GATCGTTGGG AACCGGAGCT GAATGAAGCC ATACCAAACG ACGAGCGTGA CACCACGATG
    D  R  W  E  P  E  L  N  E  A  I  P  N  D  E  R  D  T  T  M

541 CCTGCAGCAA TGGCAACAAC GTTGCGCAAA CTATTAACTG GCGAACTACT TACTCTAGCT
    P  A  A  M  A  T  T  L  R  K  L  L  T  G  E  L  L  T  L  A

601 TCCCGGCAAC AATTAATAGA CTGGATGGAG GCGGATAAAG TCGCAGGACC ACTTCTGCGC
    S  R  Q  Q  L  I  D  W  M  E  A  D  K  V  A  G  P  L  L  R
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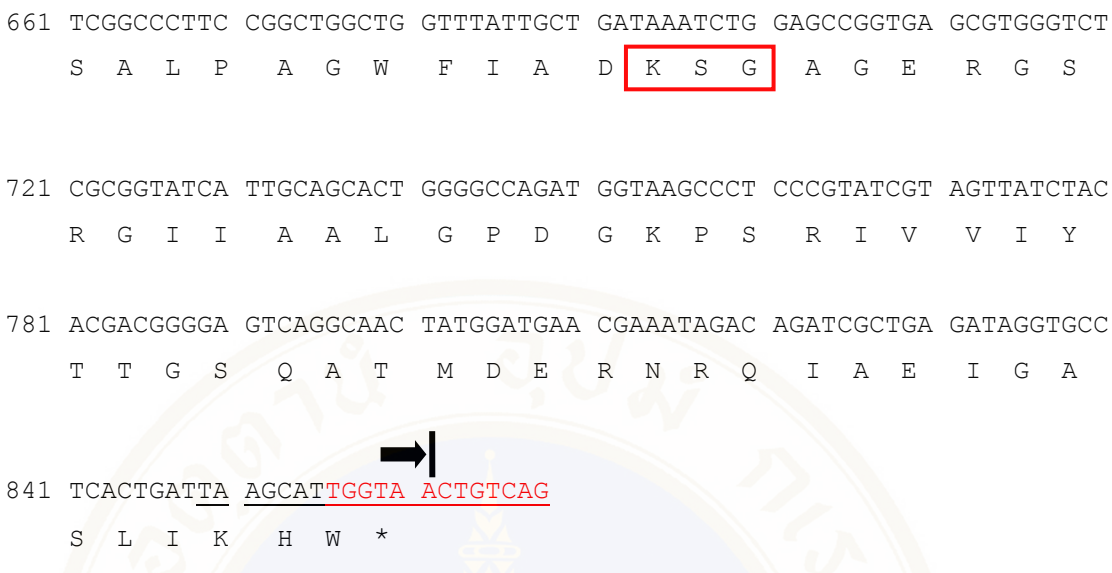

Figure 4.24 Nucleotide sequences and deduced amino acid sequence of *bla*$_{TEM}$ gene of *H. influenzae* isolate

The vertical bars indicated start or stop of the coding sequences of *bla*$_{TEM}$ gene. The underlined letters indicated sequence for primer annealing. The red letter indicated the sequence of primers which did not obtain from the real sequencing. The red boxes indicated the 4 bata-lactamase active domain

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```
                                0         10        20        30
                                |....|....|....|....|....|....|....|
E. aerogenes plasmid pYS10         MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKD
E. coli plasmid pUMB-9             MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKD
E. coli 1540 plasmid pIP1206    RKSMSIQHFRVALIPFFAAFCLPVFAHPETLVKVKD
K. pneumoniae plasmid pKpQIL       MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKD
K. pneumoniae isolate 12836        MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKD
Isolate                            MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKD
                                    40        50        60        70
                                    ....|....|....|....|....|....|....|
E. aerogenes plasmid pYS10      AEDQLGARVGYIELDLNSGKILESFRPEERFPMMS
E. coli plasmid pUMB-9          AEDQLGARVGYIELDLNSGKILESFRPEERFPMMS
E. coli 1540 plasmid pIP1206    AEDQLGARVGYIELDLNSGKILESFRPEERFPMMS
K. pneumoniae plasmid pKpQIL    AEDQLGARVGYIELDLNSGKILESFRPEERFPMMS
K. pneumoniae isolate 12836     AEDQLGARVGYIELDLNSGKILESFRPEERFPMMS
Isolate                         AEDQLGARVGYIELDLNSGKILESFRPEERFPMMS
                                      80        90       100
                                      ....|....|....|....|....|....|....|
E. aerogenes plasmid pYS10      TFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEY
E. coli plasmid pUMB-9          TFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEY
E. coli 1540 plasmid pIP1206    TFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEY
K. pneumoniae plasmid pKpQIL    TFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEY
K. pneumoniae isolate 12836     TFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEY
Isolate                         TFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEY
                                   110       120       130       140
                                   ....|....|....|....|....|....|....|
E. aerogenes plasmid pYS10      SPVTEKHLTDGMTVRELCSAAITMSDNTAANLLLT
E. coli plasmid pUMB-9          SPVTEKHLTDGMTVRELCSAAITMSDNTAANLLLT
E. coli 1540 plasmid pIP1206    SPVTEKHLTDGMTVRELCSAAITMSDNTAANLLLT
K. pneumoniae plasmid pKpQIL    SPVTEKHLTDGMTVRELCSAAITMSDNTAANLLLT
K. pneumoniae isolate 12836     SPVTEKHLTDGMTVRELCSAAITMSDNTAANLLLT
Isolate                         SPVTEKHLTDGMTVRELCSAAITMSDNTAANLLLT
                                      150       160       170
                                  ....|....|....|....|....|....|....|
E. aerogenes plasmid pYS10     TIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPN
E. coli plasmid pUMB-9         TIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPN
E. coli 1540 plasmid pIP1206   TIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPN
K. pneumoniae plasmid pKpQIL   TIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPN
K. pneumoniae isolate 12836    TIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPN
Isolate                        TIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPN
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                                 180       190       200       210
                                ....|....|....|....|....|....|....|
E. aerogenes plasmid pYS10      DERDTTMPAAMATTLRKLLTGELLTLASRQQLIDW
E. coli plasmid pUMB-9          DERDTTMPAAMATTLRKLLTGELLTLASRQQLIDW
E. coli 1540 plasmid pIP1206    DERDTTMPAAMATTLRKLLTGELLTLASRQQLIDW
K. pneumoniae plasmid pKpQIL    DERDTTMPAAMATTLRKLLTGELLTLASRQQLIDW
K. pneumoniae isolate 12836     DERDTTMPAAMATTLRKLLTGELLTLASRQQLIDW
Isolate                         DERDTTMPAAMATTLRKLLTGELLTLASRQQLIDW
                                     220       230       240
                                ....|....|....|....|....|....|....|
E. aerogenes plasmid pYS10      MEADKVAGPLLRSALPAGWFIADKSGAGERGSRGI
E. coli plasmid pUMB-9          MEADKVAGPLLRSALPAGWFIADKSGAGERGSRGI
E. coli 1540 plasmid pIP1206    MEADKVAGPLLRSALPAGWFIADKSGAGERGSRGI
K. pneumoniae plasmid pKpQIL    MEADKVAGPLLRSALPAGWFIADKSGAGERGSRGI
K. pneumoniae isolate 12836     MEADKVAGPLLRSALPAGWFIADKSGAGERGSRGI
Isolate                         MEADKVAGPLLRSALPAGWFIADKSGAGERGSRGI

                                 250       260       270       280
                                ....|....|....|....|....|....|....|
E. aerogenes plasmid pYS10      IAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEI
E. coli plasmid pUMB-9          IAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEI
E. coli 1540 plasmid pIP1206    IAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEI
K. pneumoniae plasmid pKpQIL    IAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEI
K. pneumoniae isolate 12836     IAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEI
Isolate                         IAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEI
                                     290
                                ....|....|
E. aerogenes plasmid pYS10      GASLIKHW--
E. coli plasmid pUMB-9          GASLIKHW--
E. coli 1540 plasmid pIP1206    GASLIKHW--
K. pneumoniae plasmid pKpQIL    GASLIKHW--
K. pneumoniae isolate 12836     GASLIKHW--
Isolate                         GASLIKHWLS
```

Figure 4.25 Multiple amino acid sequence alignment of one randomly selected *bla*$_{TEM}$ from *H. influenzae* with some bacteria.

Red alphabet indicated amino acid found in TEM-1 beta-lactamase.

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BLNAR *H. influenzae* isolates

1. Antimicrobial susceptibility

For 26 BLNAR *H. influenzae* isolates, the ratio of male: female patients was 1: 1. The age range was 1-80 years. The patient age groups were: <2 years (23.08%), 2-9 years (19.23%), 10-19 years (0%), 20-29 years (3.85%), 30-39 years (7.69%), 40-49 years (3.85%), 50-59 years (0%), ≥ 60 years (42.31%); most isolates were recovered from adults. These isolates tested to 9 antimicrobial agents, they were susceptible to ampicillin (0%), amoxicillin/clavulanic acid (80.77%), cefotaxime (88.46%), ceftriaxone (88.46%), ceftazidime (88.46%), cefuroxime (42.31%), ciprofloxacin (100%), tetracycline (57.69%) and trimethoprim-sulphamethoxazole (26.92%) (Table 4.24 and Fig. 4.26).

2. Correlation of BLNAR and beta-lactams

Table 4.25 shows correlation between BLNAR and beta-lactams i.e., ampicillin, amoxicillin/clavulanic acid, cefotaxime, ceftriaxone, ceftazidime and cefuroxime. 26 BLNAR isolates show 100% resistance to ampicillin. Other beta-lactams are also high susceptible e.g., cefotaxime, ceftriaxone and ceftazidime (each 88.46%), amoxicillin/clavulanic acid (80.77%) while cefuroxime (42.31%) shows less susceptibility than other drugs.

3. Prevalence of BLNAR *H. influenzae* isolates in 2008-2010

Twenty-six BLNAR isolates were found which increased from 7 isolates (3.95%) in 2008, 6 isolates (3.32%) in 2009 to 13 isolates (8.39%) in 2010 (Fig. 4.27).

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4. Determination of minimal inhibitory concentrations in BLNAR isolates

The results of 26 *H. influenzae* isolates which were negative for beta-lactamase production by nitrocefin method and ampicilln-MIC values by broth microdilution method are shown in Table 4.26 and Fig. 4.28. These isolates were determined the amoxicillin/clavulanic acid- MIC values by E-test method.

Ampicillin-MIC values 2-16 µg/ml were found in BLNAR isolates. Amoxicillin/clavulanic acid-MIC values found in range 0.75-4 µg/ml by E-test (Table 4.27 and Fig. 4.29).

5. Detection mutations in BLNAR isolates

The mutations in *ftsI* gene encoded PBP3 were detected in 10 BLNAR isolates by PCR technique. The 875 bp of *ftsI* gene fragment was amplified (Fig. 4.30). PCR products of 10 *ftsI* genes were cloned into StrataClone™ PCR cloning vector pSC-A. Then plasmids containing the PCR product insert were identified and determined insert orientation by restriction analysis by using *Eco*RI (Fig. 4.31). DNA sequences of 10 *ftsI* genes were analyzed by software available over the internet at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignment of sequences was analyzed by Bioedit program.

The MIC values and patterns of amino acid substitutions in these 10 BLNAR isolates are shown in Table 4.28.

There were 7 patterns of amino acid substitutions: isolates 1, 3; isolate 2; isolates 4, 6; isolates 5, 10; isolate 7; isolate 8; isolate 9. All isolates were group II according to Ubukata et al. (21) based on the presence of different amino acid substitution in position N526K or asparagine change to lysine and other amino acid changes in transpeptidase domain.

Patterns of amino acid substitutions in isolates 5 and 10 were correlated with *ftsI* gene in *H. influenzae* PittEE in 100% homology whereas amino acid substitution patterns in other isolates were firstly reported in this study.

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6. Restriction enzyme analysis in BLNAR *H. influenzae* isolates

The pattern of amino acid substitution in *ftsI* gene was determined by using 4 base cutter enzymes i.e., *Sau*3AI (Fig. 4.33) and *Afa*I (Fig. 4.34).

For *Sau*3AI digestion, all BLNAR isolates showed 3 different banding patterns when they were compared with control isolates (BLNAS; lanes 1 and 12). Two control isolates also gave different banding patterns. The same mutations in isolates 4 and 6 (lanes 5 and 7), gave same banding pattern. While same mutations in isolates 1 and 3 (lanes 2 and 4), gave different banding patterns. In additional, different mutations in isolates 2, 3, 4, 5, 7, 9 (lanes 3, 4, 5, 6, 8, 10) also gave same banding pattern. The results indicated that *ftsI* product digested with *Sau*3AI had variation in each isolate. Therefore mutations could not be differentiated by using this enzyme.

For *Afa*I digestion, all BLNAR isolates showed 3 different banding patterns when they were compared with control isolates (BLNAS; lanes 1 and 12). The same mutations in isolates 1 and 3 (lanes 2 and 4), isolates 4 and 6 (lanes 5 and 7), isolates 5 and 10 (lanes 6 and 11) gave same banding pattern. The different mutations in isolates 1, 2 and 9 (lanes 2, 3 and 10), gave difference in banding patterns but other different mutations in isolates 1, 4, 5, 7, 8 (lanes 2, 5, 6, 8, 9) gave same banding pattern. In additional, all BLNAR isolates also differed from control isolates. Although some BLNAR isolates with different mutations showed same banding pattern, they also differed from control isolates. Therefore *Afa*I enzyme may roughly group these isolates.

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Table 4.24 Antimicrobial susceptibility of BLNAR *H. influenzae* by disk diffusion method

Antimicrobial agents	Number (%) of susceptibility		
	Susceptible	Intermediate	Resistance
Ampicillin	-	-	26 (100)
Amoxicillin/clavulanic acid	21 (80.77)	-	5 (19.23)
Cefotaxime	23 (88.46)	-	3 (11.54)
Ceftriaxone	23 (88.46)	-	3 (11.54)
Ceftazidime	23 (88.46)	2 (7.69)	1 (3.85)
Cefuroxime	11 (42.31)	3 (11.54)	12 (46.15)
Ciprofloxacin	26 (100)	-	-
Tetracycline	15 (57.69)	4 (15.39)	7 (26.92)
Trimethoprim-sulphamethoxazole	7 (26.92)	1 (3.85)	18 (69.23)

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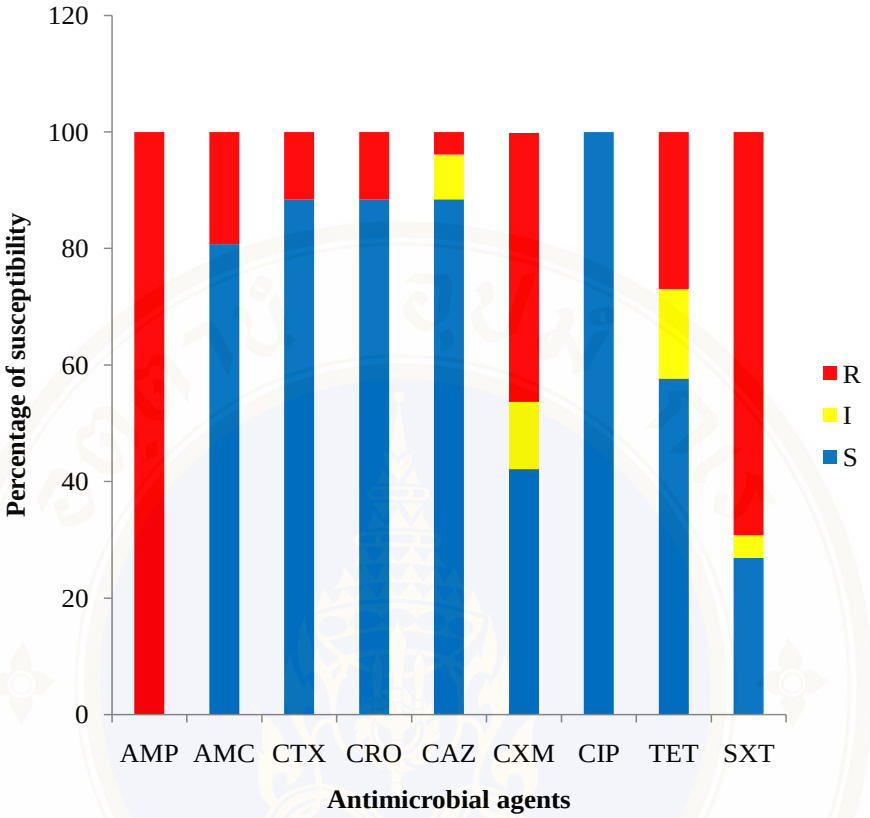

Figure 4.26 Percentage of antimicrobial susceptibility of BLNAR *H. influenzae*

Abbreviation: AMP = ampicillin, AMC = amoxicillin/clavulanic acid, CTX = cefotaxime, CRO = ceftriaxone, CAZ = ceftazidime, CXM = cefuroxime, CIP = ciprofloxacin, TET = tetracycline and SXT = trimethoprim-sulphamethoxazole. S = susceptible, I = intermediate, R = resistant.

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Table 4.25 Correlation of BLNAR and beta-lactam susceptibility in 26 BLNAR isolates

Beta-lactams	S (%)	I (%)	R (%)
AMP	-	-	100
AMC	80.77	-	19.23
CTX	88.46	-	11.54
CRO	88.46	-	11.54
CAZ	88.46	7.69	3.85
CXM	42.31	11.54	46.15

Abbreviation: AMP = ampicillin, AMC = amoxicillin/clavulanic acid, CTX = cefotaxime, CRO = ceftriaxone, CAZ = ceftazidime, CXM = cefuroxime, CIP = ciprofloxacin, TET = tetracycline and SXT = trimethoprim-sulphamethoxazole. S = susceptible, I = intermediate, R = resistant.

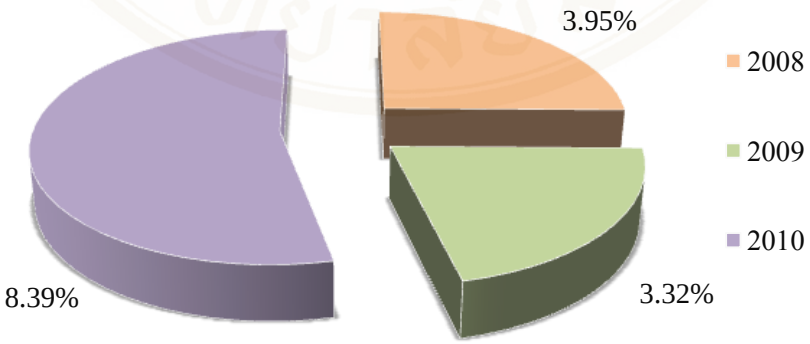

Figure 4.27 Prevalence of BLNAR *H. influenzae* isolates in 2008-2010

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Table 4.26 Minimal inhibitory concentrations of ampicillin in BLNAR isolates

	Minimal inhibitory concentrations (µg/ml)			
	2	4	8	16
No. of isolates	15	8	2	1

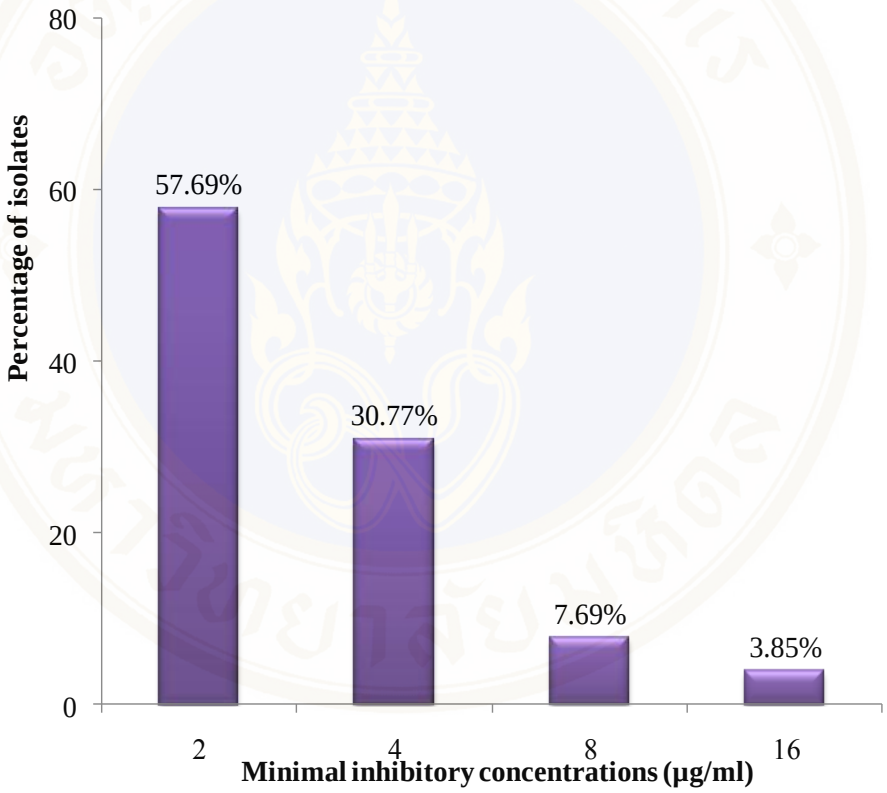

Figure 4.28 Minimal inhibitory concentrations of ampicillin in BLNAR isolates

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Table 4.27 Minimal inhibitory concentrations of amoxicillin/clavulanic acid in BLNAR isolates

	Minimal inhibitory concentrations (µg/ml)					
No. of isolates	0.75	1	1.5	2	3	4
	2	2	1	6	10	5

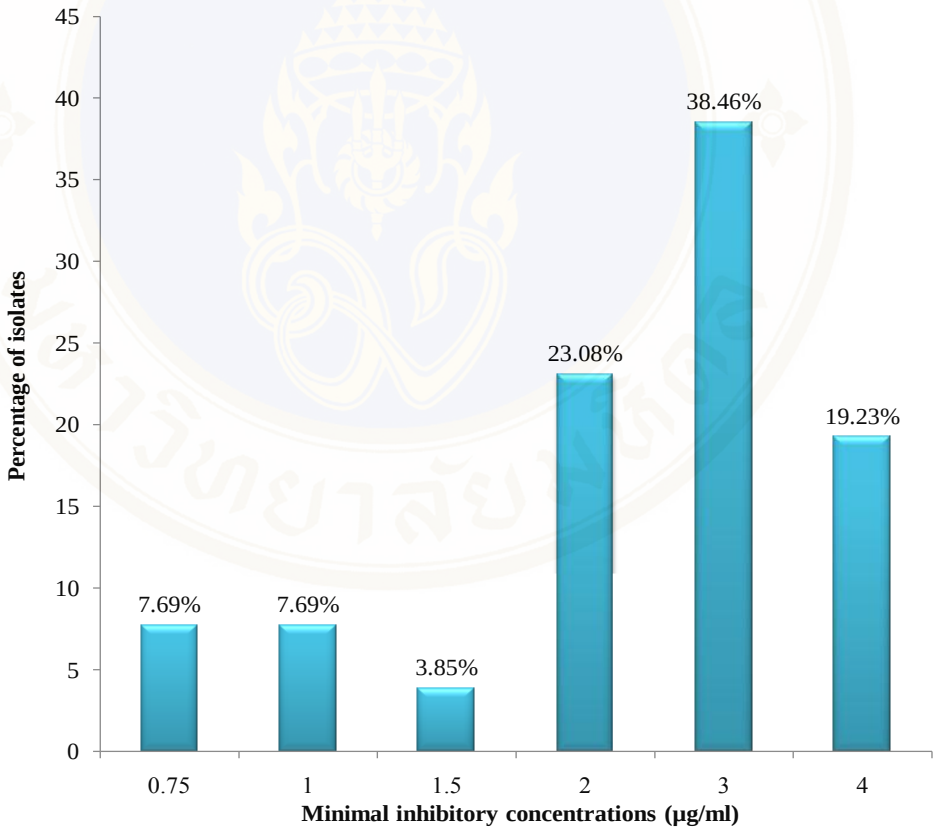

Figure 4.29 Minimal inhibitory concentrations of amoxicillin/clavulanic acid in BLNAR isolates

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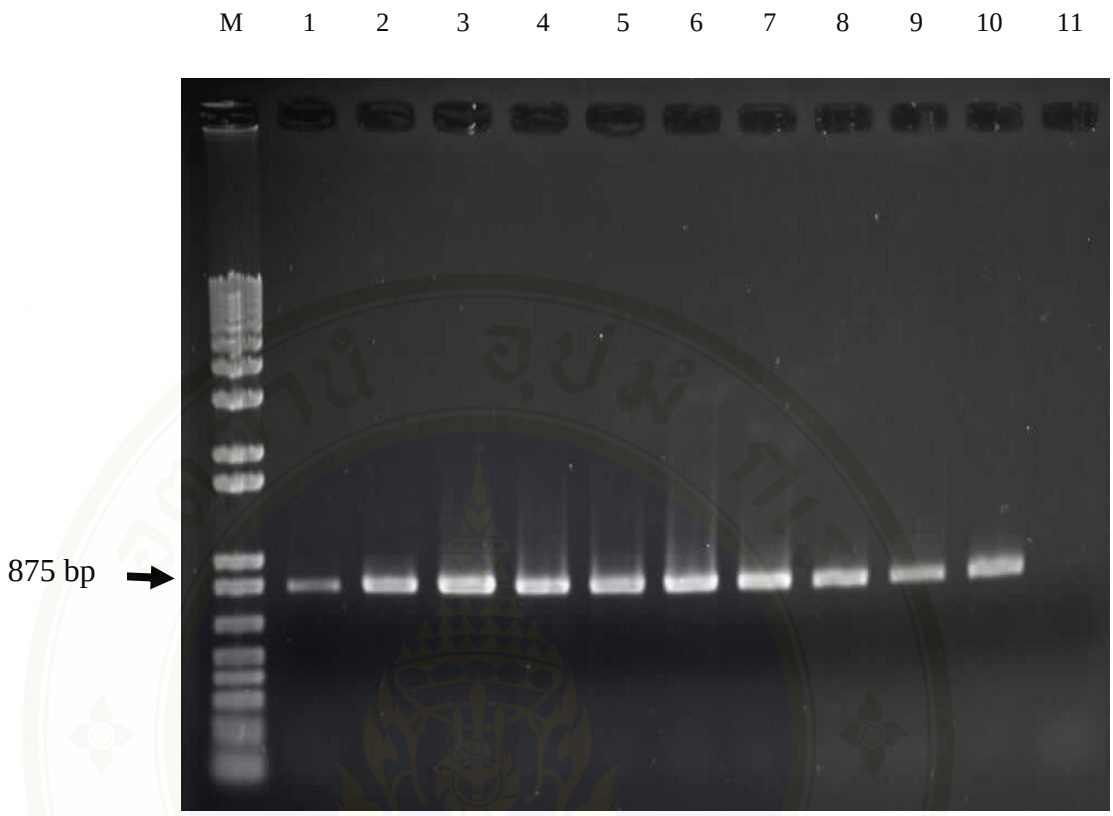

Figure 4.30 PCR products of 875 bp *ftsI* genes fragment of *H. influenzae*
Lane M was the standard 1 kb plus DNA size marker. Lanes 1-10 were *ftsI* gene carrying 875 bp fragment of *ftsI* positive. Lane 11 was negative control.

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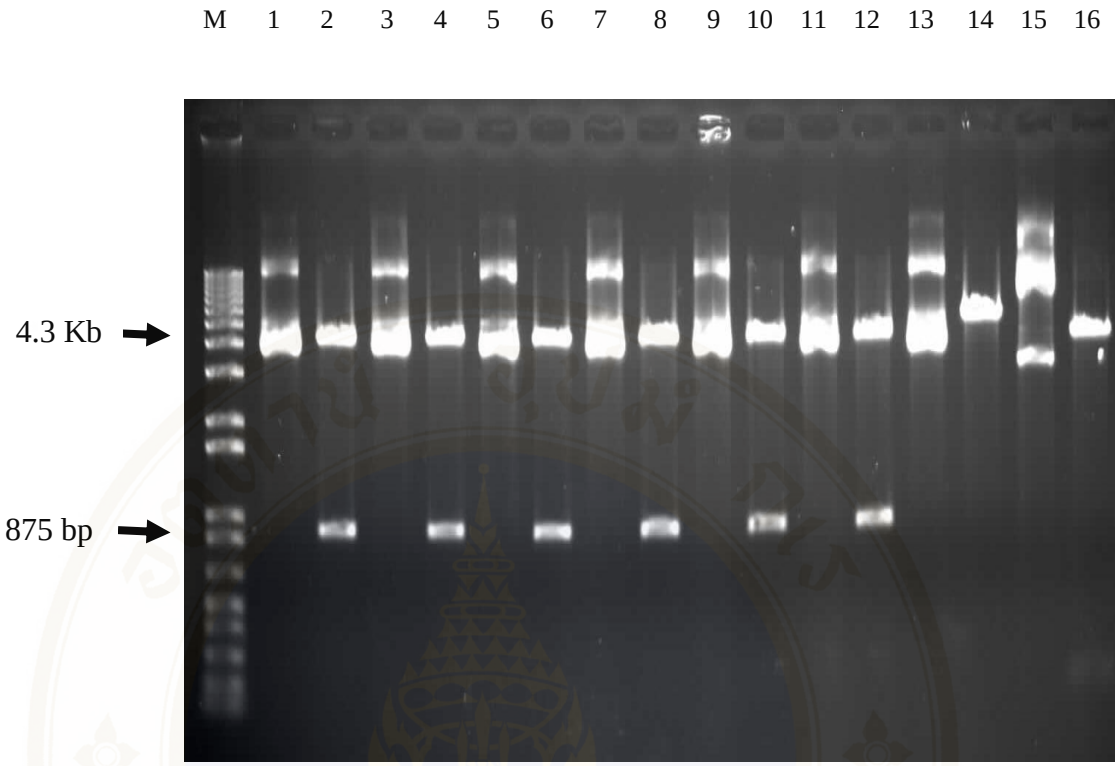

Figure 4.31 Insertion of plasmids containing PCR product (*ftsI*) by *Eco*RI restriction analysis

Lane M was the standard 1 kb plus DNA size marker. Lanes 1, 3, 5, 7, 9, 11 = undigested plasmids containing PCR product of *ftsI* gene insertion. Lanes 2, 4, 6, 8, 10, 12 = digested plasmids containing PCR product of *ftsI* gene insertion. Lanes 13, 15 = undigested plasmids without PCR product of *ftsI* gene insertion. Lanes 14, 16 = digested plasmids without PCR product of *ftsI* gene insertion.

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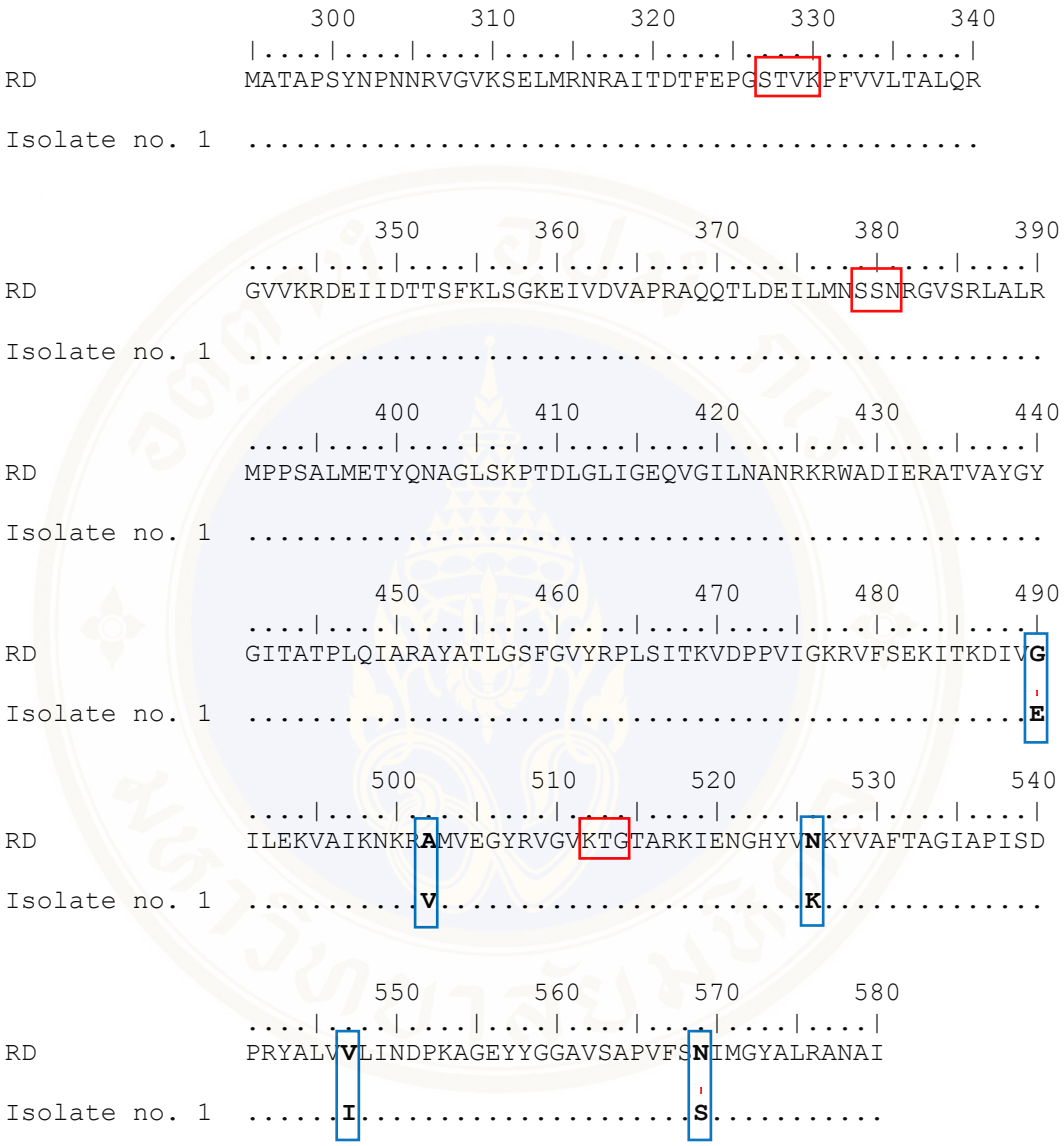
Isolate no. 1



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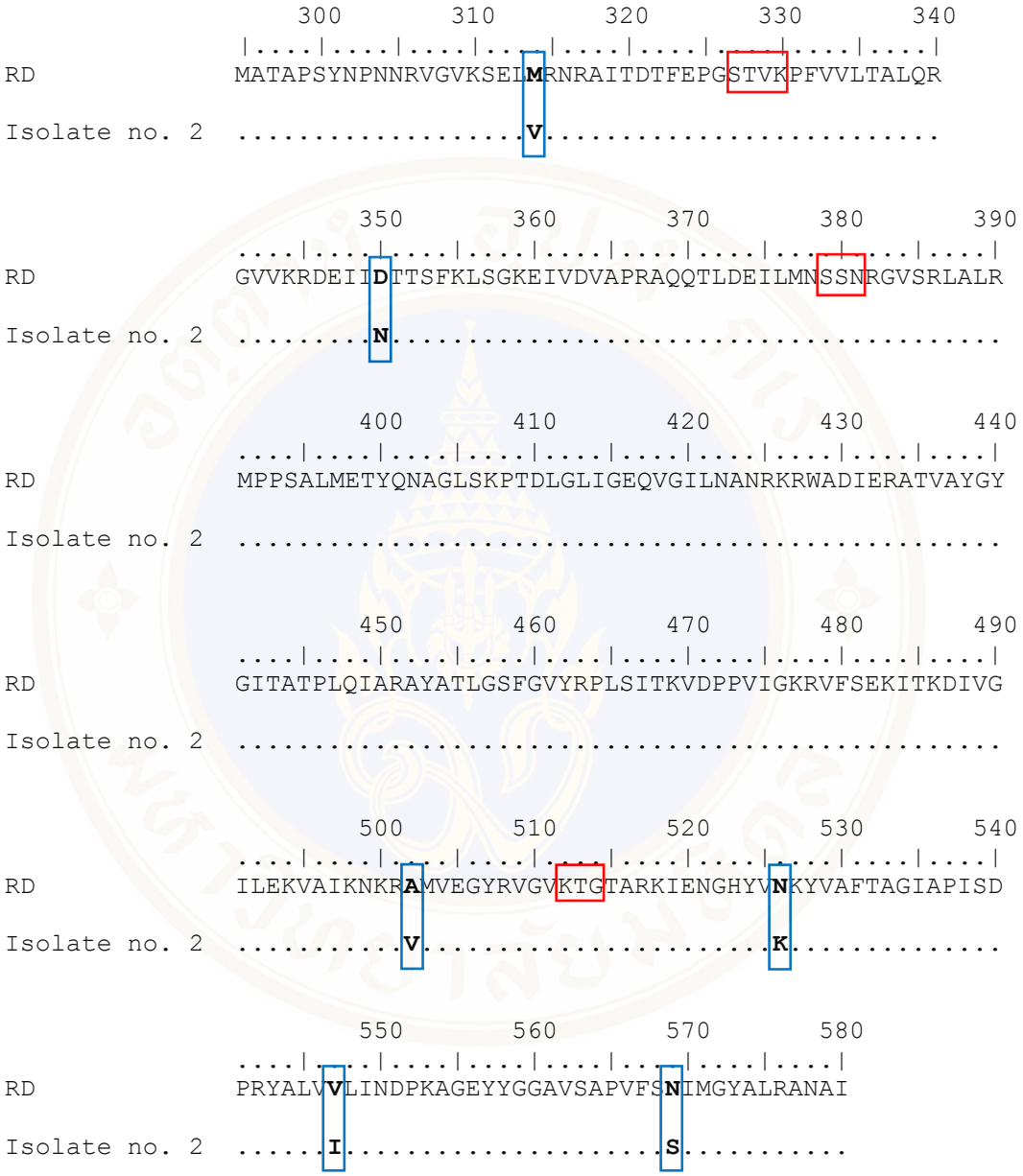
Isolate no. 2



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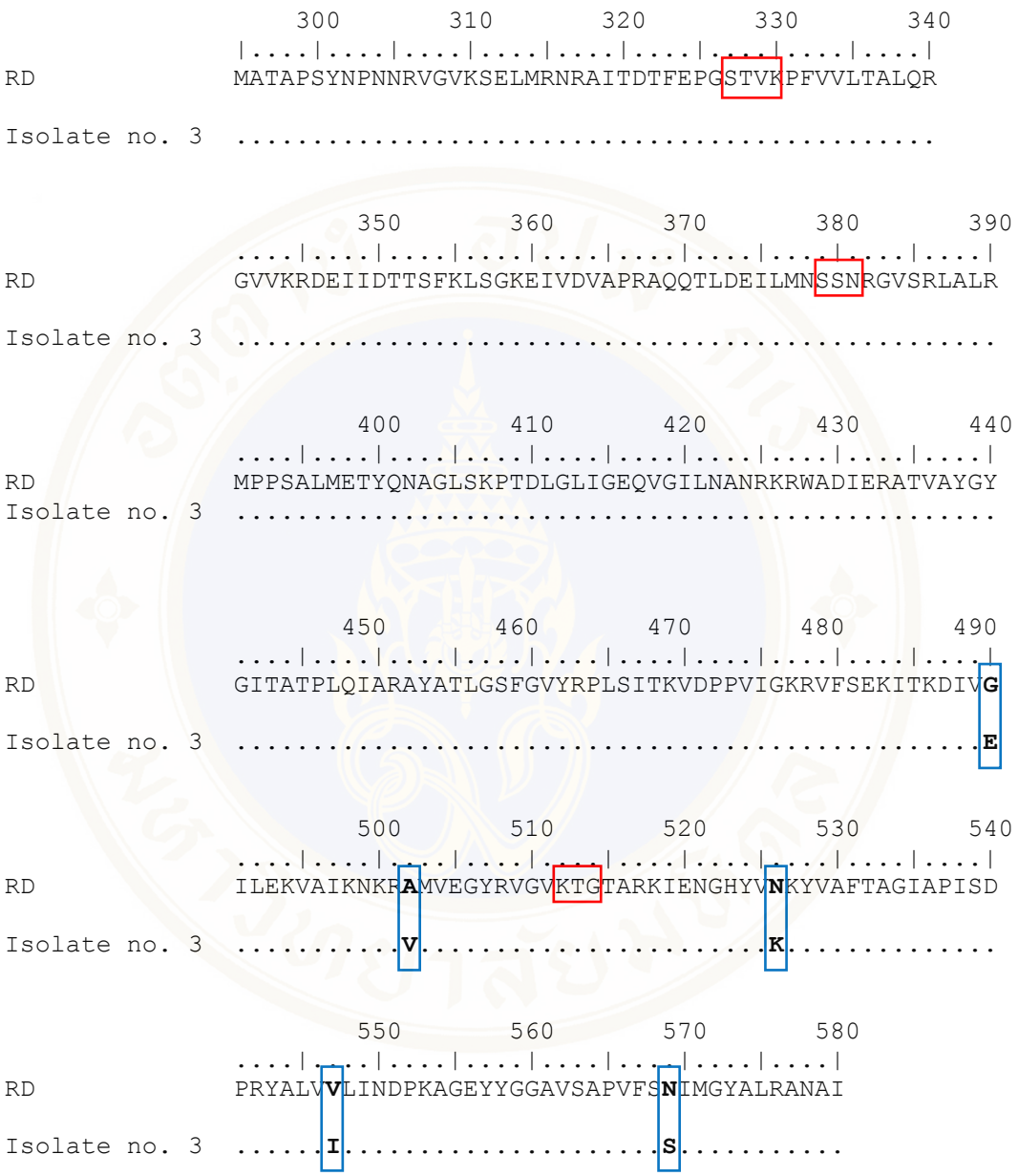

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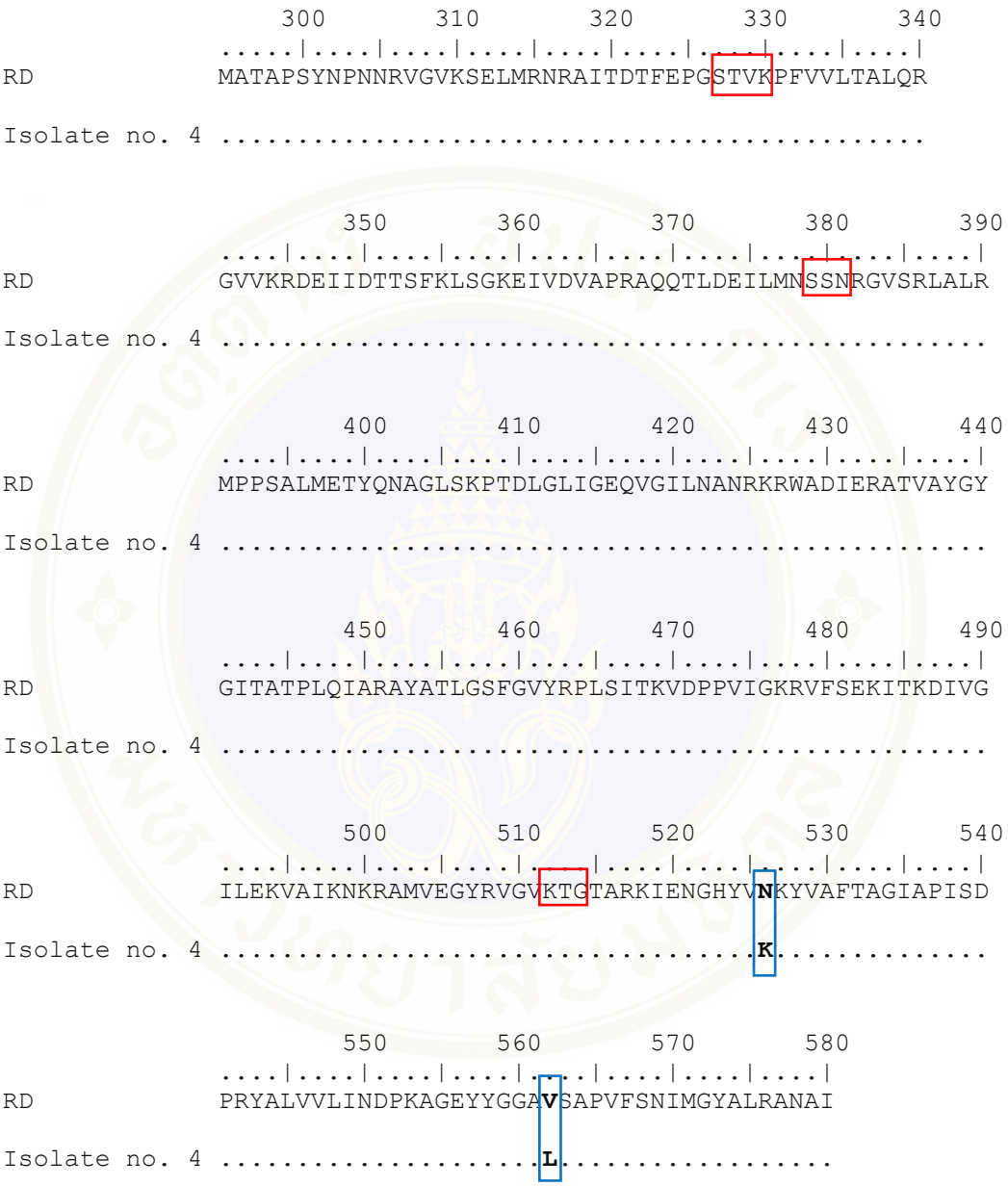
Isolate no. 4



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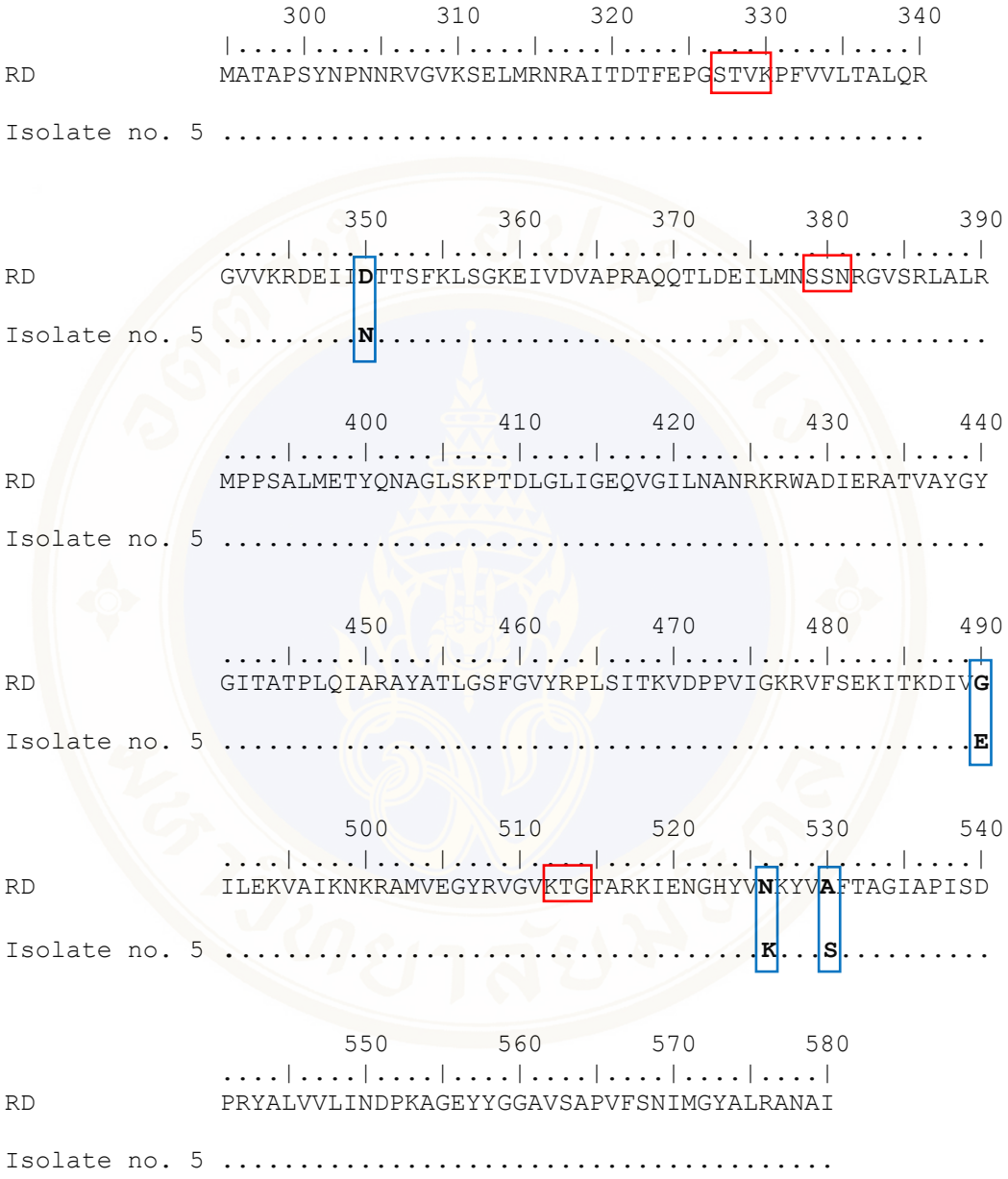

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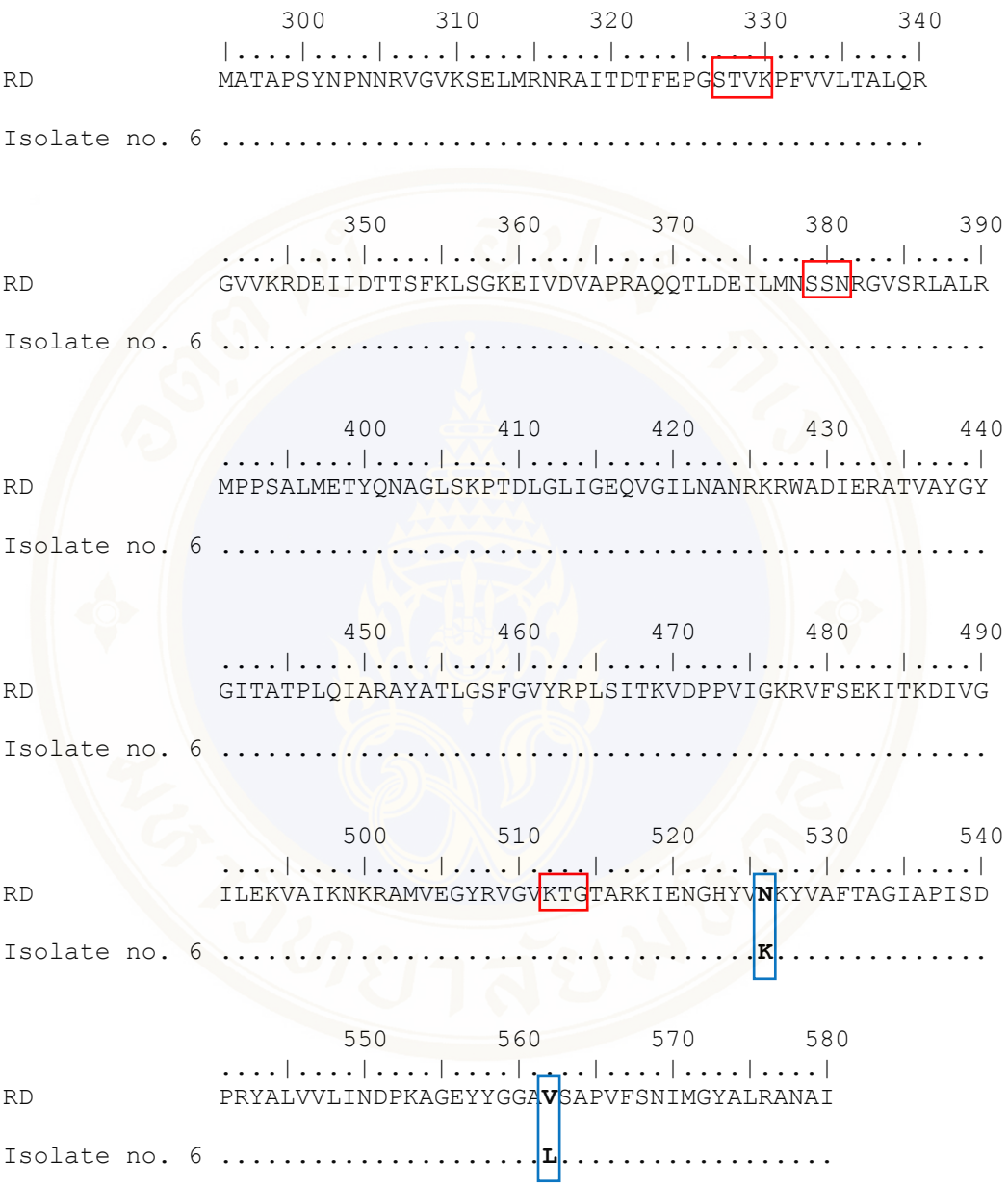
Isolate no. 6



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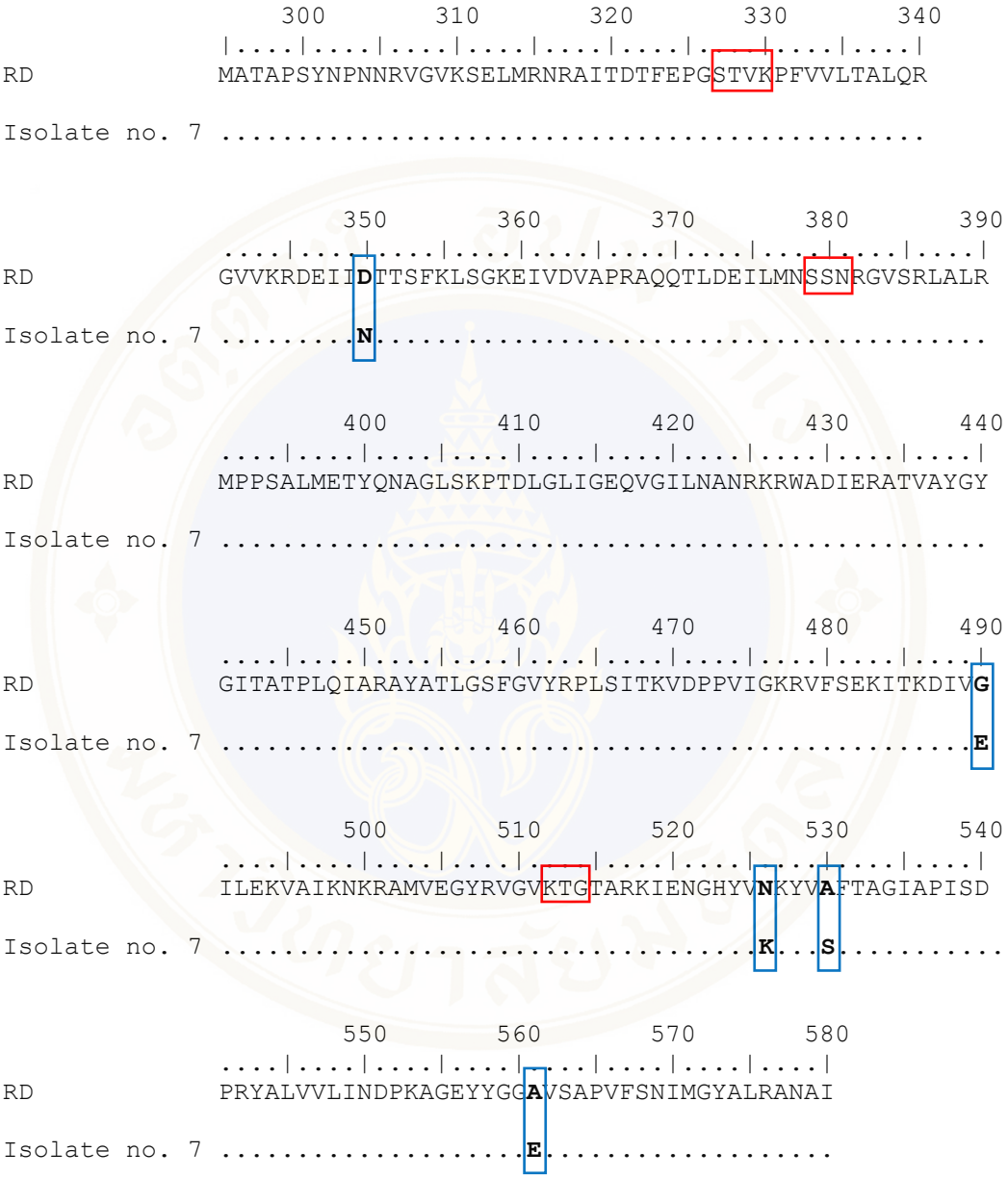
Isolate no. 7



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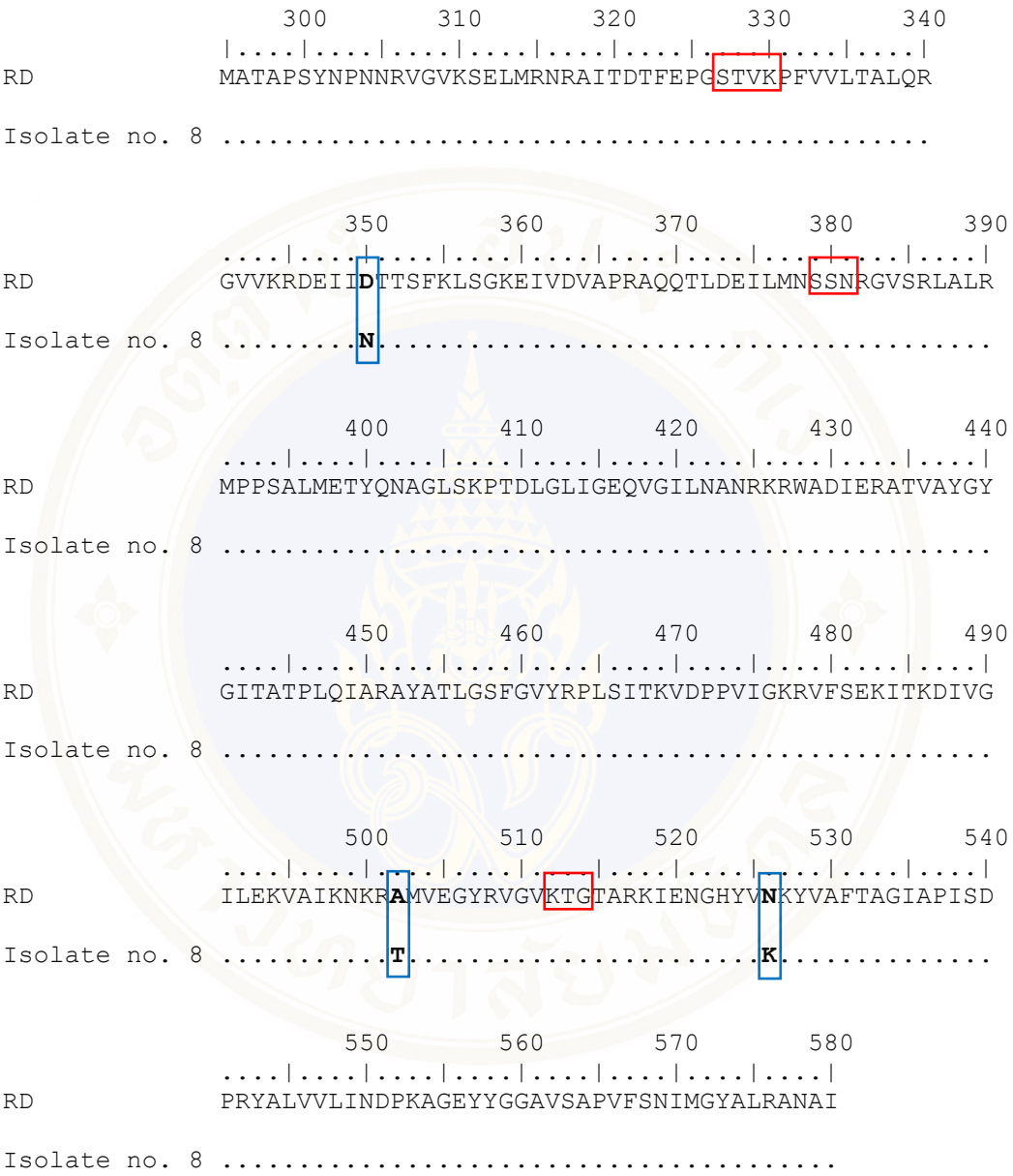
Isolate no. 8



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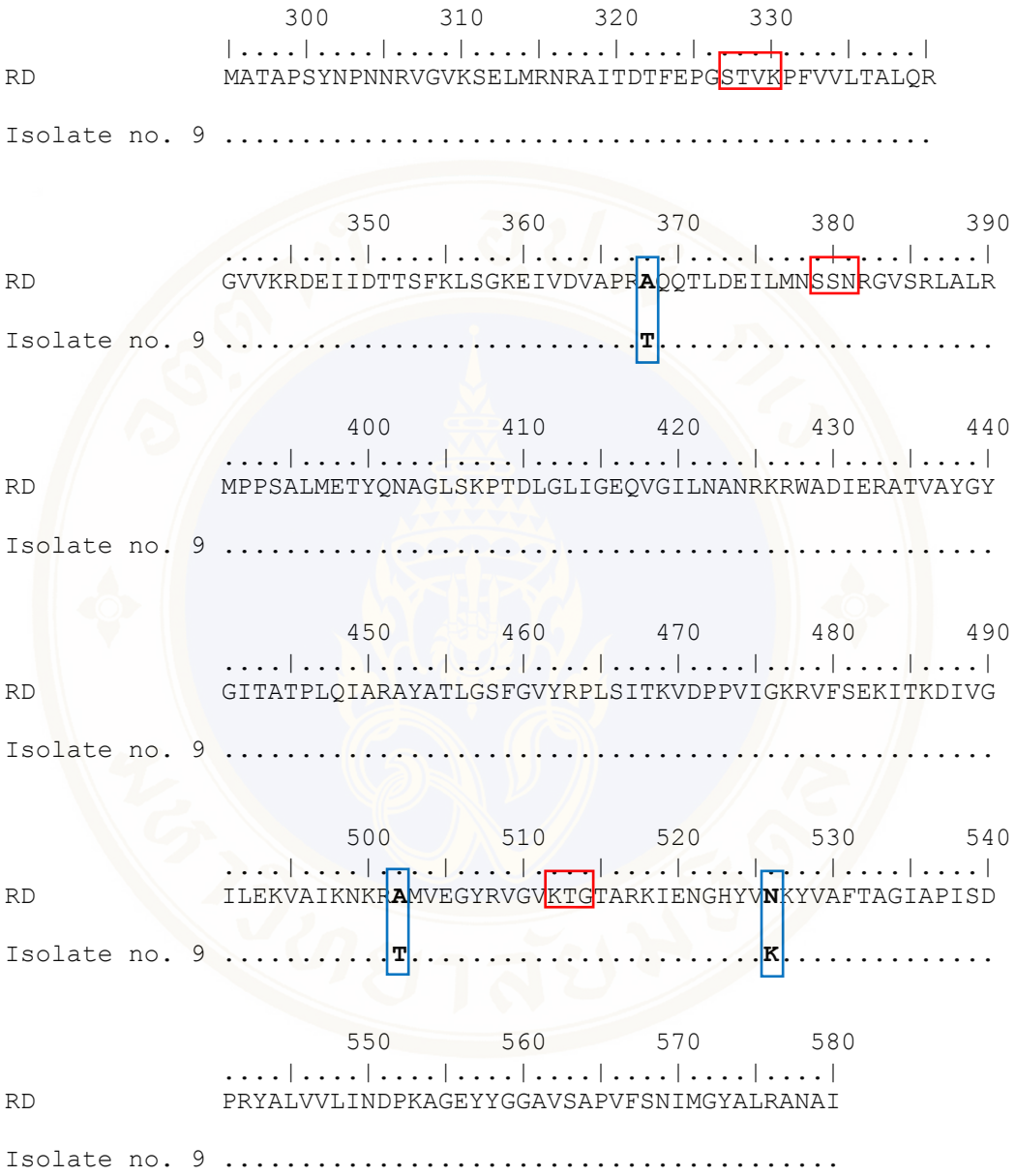
Isolate no. 9



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Isolate no. 10

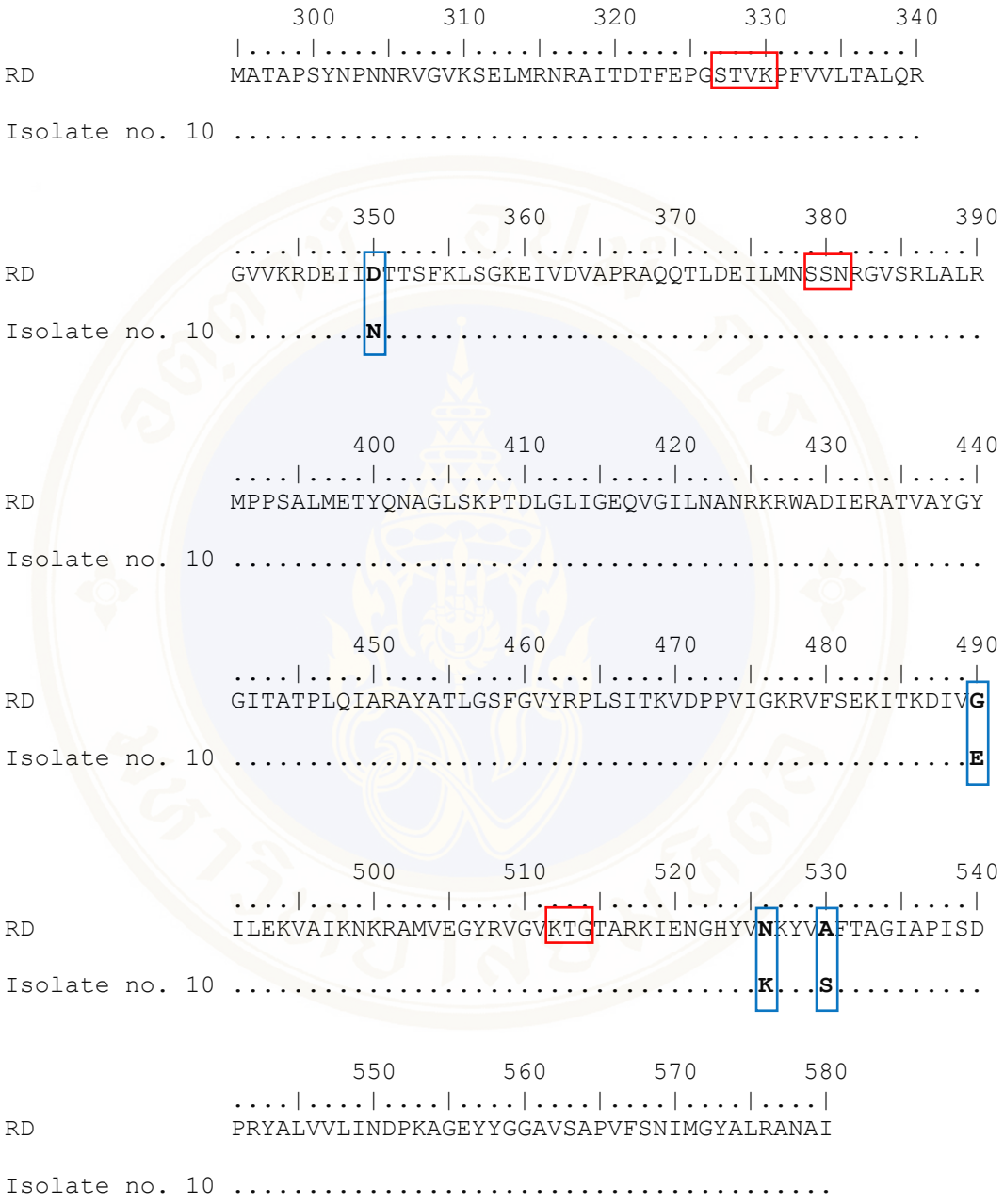

Figure 4.32 Amino acid sequence alignment of translated *ftsI* protein from *H. influenzae* Rd KW20 (GenBank accession no. NC_000907) and isolates No. 1-10, respectively. Red boxes indicate three conserved motifs in the active site. Dots indicate identical amino acids and blue boxes indicate amino acid substitution.

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Table 4.28 Minimal inhibitory concentrations and amino acid substitutions

No. of isolates	MIC (µg/ml)	Amino acid at positions:										
		314	**350**	**368**	**490**	**502**	**526**	**530**	**547**	**561**	**562**	**569**
1	2				E	V	K		I			S
2	2	V	N			V	K		I			S
3	2				E	V	K		I			S
4	4						K				L	
5	8		N		E		K	S				
6	4						K				L	
7	16		N		E		K	S		E		
8	2		N			T	K					
9	2			T		T	K					
10	8		N		E		K	S				

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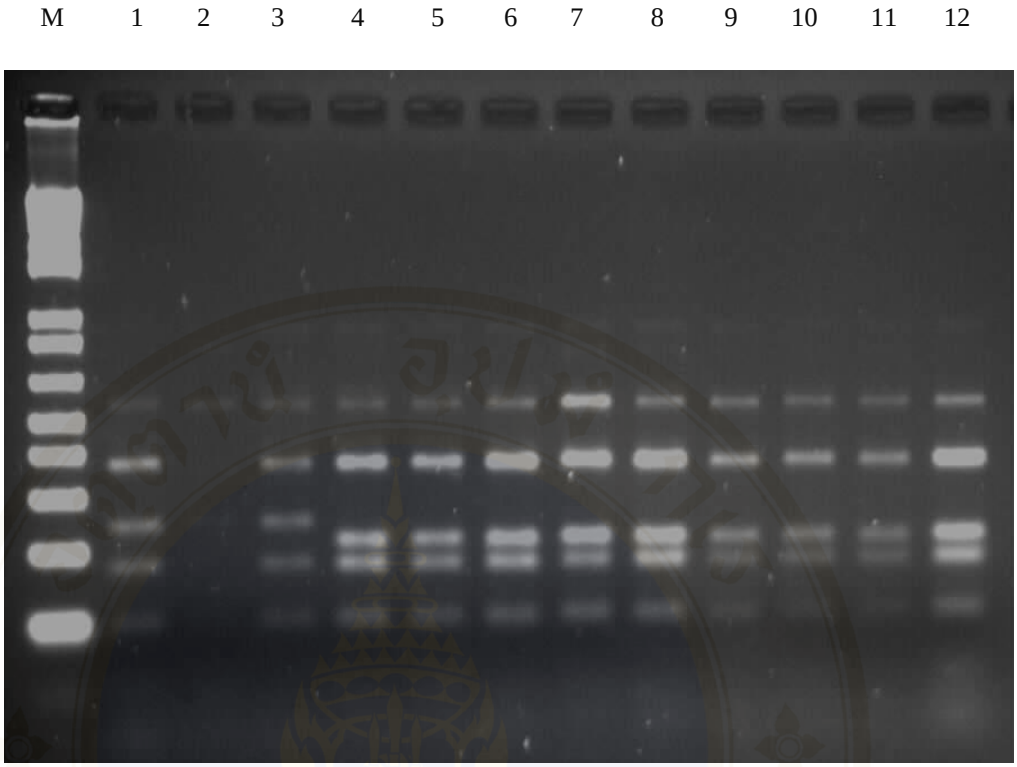

Figure 4.33 Pattern of *ftsI* gene digested with *Sau*3AI enzyme

Lane M was the standard 1 kb plus DNA size marker. Lanes 1, 12 were 2 isolates of beta-lactamase negative ampicilln susceptible (BLNAS). Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 were isolates no. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, respectively.

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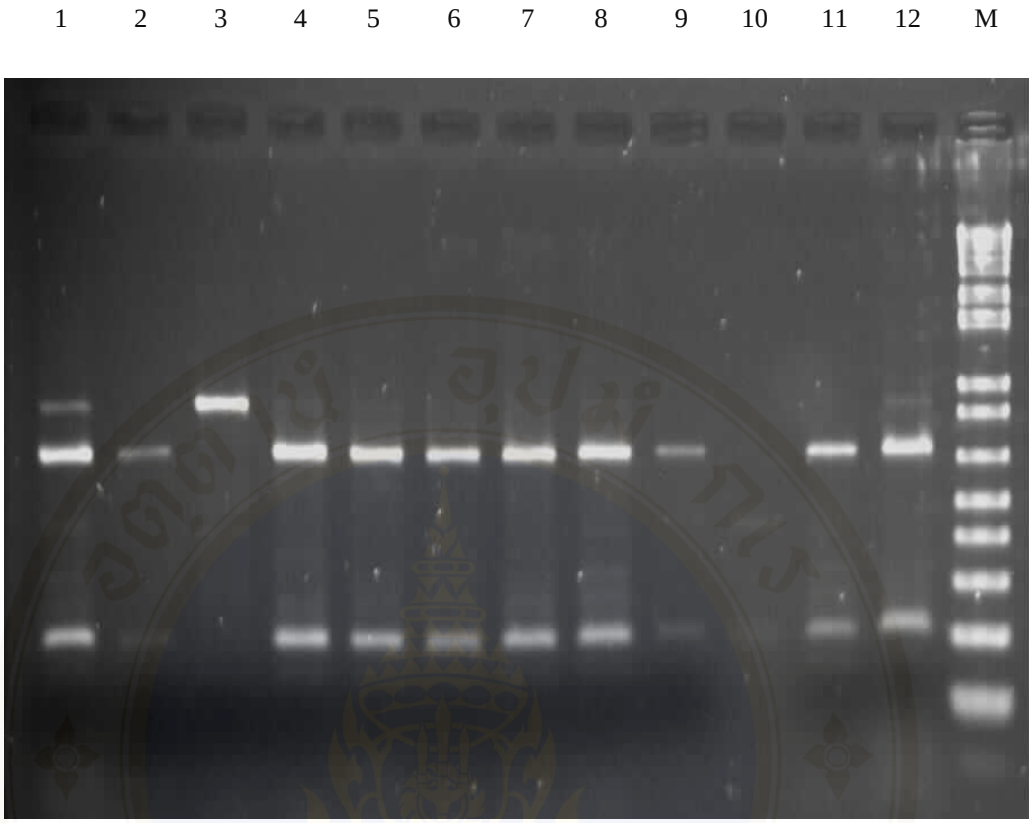

Figure 4.34 Pattern of *ftsI* gene digested with *Afa*I enzyme

Lane M was the standard 1 kb plus DNA size marker. Lanes 1, 12 were 2 isolates of beta-lactamase negative ampicilln susceptible (BLNAS). Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 were isolates no. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, respectively

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Essential oils

The result of gas chromatography analysis found the most constituent in kaffir lime leaf oil was citronellal 80.04%, whereas kaffir lime oil contained 40.65% of limonene, 13.71% of terpinene-4-ol and 13.20% of alpha-terpineol.

The result of kaffir lime leaf oil and kaffir lime oil to *H. influenzae* by disk diffusion and broth microdilution methods was shown in Tables 4.29 and 4.30.

Kaffir lime leaf oil and kaffir lime oil showed high antibacterial activities to other respiratory tract pathogen i.e., Group A streptococci (Tables 4.31 and 4.32), Group B streptococci (Tables 4.33 and 4.34), Group C streptococci (Tables 4.35 and 4.36), Group F streptococci (Tables 4.37 and 4.38), Group G streptococci (Tables 4.39 and 4.40), *S. pneumoniae* (Tables 4.41 and 4.42), *S. aureus* (MRSA) (Tables 4.43 and 4.44), *S. aureus* (MSSA) (Tables 4.45 and 4.46) and *A. baumannii* (Tables 4.47 and 4.48) by both disk diffusion and broth microdilution methods. The overall results are summarized in Fig 4.37, Tables 4.49 and 4.50.

For kaffir lime leaf oil, the large inhibition zones for these pathogens in decreasing order were found in *H. influenzae*, *S. pneumoniae*, Group A, Group F, Group G, Group C, Group B streptococci, *S. aureus* (MRSA, MSSA) and *A. baumannii*, respectively. While large inhibition zones from kaffir lime oil in decreasing order were found in *S. aureus* (MRSA, MSSA), *A. baumannii*, *S. pneumoniae*, Group F streptococci, *H. influenzae*, Group A, Group G, Group C and Group B streptococci, respectively. Therefore, both kaffir lime leaf oil and kaffir lime oil exhibited antibacterial properties when they were screened by disk diffusion.

From MIC and MBC values of kaffir lime leaf oil and kaffir lime oil, *H. influenzae* gave the lowest MIC for kaffir lime leaf oil and kaffir lime oil than other tested bacteria. Interestingly, bacteria tested showed low MIC values, when tested with kaffir lime oil although some of bacteria tested showed large inhibition zone in experiment with kaffir lime leaf oil.

In addition, the major component in kaffir lime leaf oil is citronellal. The latter was tested and showed good antibacterial activity (Table 4.51). Citronellal had similar activity with kaffir lime leaf oil in all tested bacteria, therefore it may be the active ingredient in kaffir lime leaf oil. While one component in kaffir lime oil is limonene which was tested to prove the antibacterial activity. However, it showed

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poor antibacterial activity compared with kaffir lime oil in all bacteria tested (Table 4.52). Alpha-terpineol is another component found in kaffir lime oil in additional to limonene. Interestingly, alpha-terpineol indicated a capability of a very high antibacterial activity against all bacteria tested (Table 4.53). Alpha-terpineol's inhibitory effect is much higher than that of kaffir lime oil. Therefore kaffir lime oil may show antibacterial activity from minor component or it needs other components e.g., alpha-terpineol for the synergistic effect.

Stability of kaffir lime leaf oil and kaffir lime oil was also tested for 12% kaffir lime leaf oil and 10% kaffir lime oil. These 2 oils were adjusted their odor in order to obtain a proper use by reducing the percentage of oils and addition of other odors e.g., 12% kaffir lime 1eaf oil was added with blueberry odor while 10% kaffir lime oil was added with pineapple odor. This experiment was tested with Group A streptococci and 3 standard reference isolates; *S. pneumoniae* ATCC 49619, *S. aureus* ATCC 29213 and *H. influenzae* ATCC 49766 by broth microdilution method. These results were shown in Tables 4.53 and 4.54.

For 12% kaffir lime leaf oil, all isolates tested during 4 months did not show significant difference in MIC and MBC values when they were compared with antibacterial activity in 1, 2 and 3 months. In addition, all isolates tested did not show significant difference in MIC and MBC values when they were tested with 10% kaffir lime oil. From these results, oils of kaffir lime were still stable during 4 months storage at room temperature.

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Table 4.29 Disk diffusion of kaffir lime leaf oil and kaffir lime oil to *H. influenzae* (52 isolates)

Lab No.	Kaffir lime leaf oil (mm)	Kaffir lime oil (mm)
1	21	17
2	27	16
3	27	16
4	30	19
5	28	16
6	30	18
7	25	14
8	35	17
9	30	15
10	46	20
11	37	16
12	34	17
13	35	23
14	32	22
15	46	18
16	22	13
17	25	17
18	23	15
19	37	16
20	20	16
21	22	12
22	28	14
23	22	15
24	29	16
25	21	14
26	34	14
27	34	14
28	30	16
29	25	16
30	29	12
31	22	14
32	28	15
33	24	18
34	38	17
35	23	13
36	39	16

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Table 4.29 Disk diffusion of kaffir lime leaf oil and kaffir lime oil to *H. influenzae* (52 isolates)

Lab No.	Kaffir lime leaf oil (mm)	Kaffir lime oil (mm)
37	25	14
38	25	16
39	38	20
40	33	16
41	32	14
42	33	13
43	30	20
44	27	17
45	32	15
46	20	17
47	28	17
48	28	17
49	27	17
50	27	15
51	33	14
52	21	14
Mean±S.D.	29.17±6.18	16.02±2.3

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Table 4.30 MIC and MBC values of kaffir lime leaf oil and kaffir lime oil to *H. influenzae* (52 isolates)

Lab No.	Kaffir lime leaf oil (mg/ml)		Kaffir lime oil (mg/ml)	
	MIC	MBC	MIC	MBC
1	0.2	0.2	0.3	0.3
2	0.2	0.2	0.06	0.1
3	0.2	0.2	0.3	0.3
4	0.3	0.3	0.5	0.5
5	0.06	0.06	0.06	0.06
6	0.3	0.3	0.3	0.5
7	0.5	1.1	0.6	1.1
8	0.5	0.5	0.3	0.3
9	0.3	0.5	0.1	0.1
10	0.3	0.3	0.1	0.1
11	0.06	0.2	0.1	0.1
12	0.2	0.2	0.1	0.3
13	0.2	0.3	0.1	0.3
14	0.3	0.3	0.3	0.3
15	0.2	0.2	0.1	0.3
16	0.06	0.2	0.06	0.1
17	0.3	0.3	0.1	0.3
18	0.06	0.06	0.06	0.1
19	0.06	0.06	0.06	0.06
20	0.2	0.2	0.1	0.1
21	0.06	0.2	0.1	0.1
22	0.2	0.2	0.1	0.3
23	0.2	0.2	0.06	0.1
24	0.2	0.2	0.1	0.1
25	0.06	0.2	0.06	0.3
26	0.06	0.2	0.06	0.1
27	0.3	0.3	0.1	0.3
28	0.2	0.3	0.06	0.06
29	0.2	0.2	0.1	0.1
30	0.3	0.3	0.3	0.3
31	0.2	0.2	0.3	0.3
32	0.06	0.06	0.3	0.1
33	0.2	0.2	0.1	0.1
34	0.2	0.2	0.3	0.3

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Table 4.30 MIC and MBC values of kaffir lime leaf oil and kaffir lime oil to *H. influenzae* (52 isolates)

Lab No.	Kaffir lime leaf oil (mg/ml)		Kaffir lime oil (mg/ml)	
	MIC	MBC	MIC	MBC
35	0.2	0.2	0.06	0.1
36	0.3	0.3	0.3	0.5
37	0.2	0.3	0.06	0.1
38	0.06	0.06	0.1	0.3
39	0.06	0.06	0.06	0.06
40	0.06	0.2	0.1	0.3
41	0.2	0.2	0.3	0.3
42	0.3	0.3	0.1	0.1
43	0.2	0.3	0.06	0.1
44	0.2	0.3	0.1	0.1
45	0.2	0.3	0.06	0.1
46	0.2	0.3	0.06	0.06
47	0.3	0.3	0.3	0.3
48	0.06	0.2	0.1	0.3
49	0.2	0.2	0.3	0.3
50	0.3	0.3	0.3	0.3
51	0.2	0.2	0.1	0.1
52	0.3	0.3	0.3	0.5

Kaffir lime leaf oil

MIC range: 0.06-0.5 mg/ml

MIC_{50} = 0.2 mg/ml

MIC_{90} = 0.3 mg/ml

MBC range: 0.06-1.1 mg/ml

Kaffir lime oil

MIC range: 0.06-0.5 mg/ml

MIC_{50} = 0.1 mg/ml

MIC_{90} = 0.3 mg/ml

MBC range: 0.06-1.1 mg/ml

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Table 4.31 Disk diffusion of kaffir lime leaf oil and kaffir lime oil to Group A streptococci (61 isolates)

Lab No.	Kaffir lime leaf oil (mm)	Kaffir lime oil (mm)
1	16	14
2	19	14
3	23	14
4	18	15
5	18	14
6	19	16
7	20	16
8	18	14
9	19	16
10	32	23
11	16	11
12	17	13
13	17	14
14	18	14
15	19	13
16	17	15
17	19	14
18	21	14
19	18	15
20	19	13
21	17	13
22	18	13
23	19	14
24	18	15
25	18	14
26	20	15
27	19	14
28	19	15
29	17	13
30	15	12
31	17	14
32	16	13
33	19	14
34	19	15
35	16	14

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Table 4.31 Disk diffusion of kaffir lime leaf oil and kaffir lime oil to Group A streptococci (61 isolates)

Lab No.	Kaffir lime leaf oil (mm)	Kaffir lime oil (mm)
36	16	13
37	19	14
38	20	15
39	20	14
40	19	15
41	17	13
42	17	14
43	19	14
44	17	13
45	18	15
46	19	14
47	18	13
48	29	14
49	29	17
50	17	14
51	19	15
52	17	13
53	18	14
54	20	16
55	18	13
56	20	15
57	21	17
58	15	12
59	21	16
60	17	12
61	20	15
Mean±S.D.	18.85±3	14.26±1.64

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Table 4.32 MIC and MBC values of kaffir lime leaf oil and kaffir lime oil to Group A streptococci (61 isolates)

Lab No.	Kaffir lime leaf oil (mg/ml)		Kaffir lime oil (mg/ml)	
	MIC	MBC	MIC	MBC
1	4.3	4.3	2.2	2.2
2	2.1	2.1	1.1	1.1
3	1.1	4.3	1.1	1.1
4	1.1	2.1	0.5	0.5
5	4.3	4.3	1.1	1.1
6	1.1	1.1	0.5	0.5
7	1.1	1.1	1.1	1.1
8	2.1	2.1	1.1	1.1
9	4.3	4.3	2.2	2.2
10	4.3	4.3	2.2	2.2
11	1.1	1.1	1.1	1.1
12	2.1	2.1	2.2	2.2
13	2.1	2.1	1.1	1.1
14	1.1	1.1	1.1	1.1
15	1.1	1.1	0.5	0.5
16	1.1	2.1	1.1	1.1
17	0.5	0.5	0.5	0.5
18	1.1	1.1	0.5	0.5
19	2.1	4.3	1.1	1.1
20	2.1	2.1	1.1	1.1
21	0.3	0.3	0.3	0.3
22	2.1	2.1	2.2	2.2
23	1.1	1.1	1.1	1.1
24	0.5	0.5	0.5	0.5
25	1.1	1.1	1.1	1.1
26	1.1	1.1	1.1	1.1
27	2.1	2.1	2.2	2.2
28	1.1	1.1	1.1	1.1
29	8.5	8.5	2.2	2.2
30	2.1	4.3	4.4	4.4
31	2.1	4.3	1.1	2.2
32	0.5	0.5	0.5	0.5
33	2.1	2.1	2.2	2.2
34	4.3	4.3	2.2	2.2
35	2.1	2.1	1.1	1.1

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Table 4.32 MIC and MBC values of kaffir lime leaf oil and kaffir lime oil to Group A streptococci (61 isolates)

Lab No.	Kaffir lime leaf oil (mg/ml)		Kaffir lime oil (mg/ml)	
	MIC	MBC	MIC	MBC
36	4.3	4.3	1.1	1.1
37	2.1	2.1	2.2	2.2
38	4.3	4.3	4.4	4.4
39	4.3	4.3	2.2	4.4
40	4.3	4.3	2.2	4.4
41	4.3	4.3	2.2	4.4
42	4.3	4.3	2.2	2.2
43	1.1	1.1	1.1	1.1
44	0.3	0.3	0.3	0.3
45	1.1	1.1	1.1	1.1
46	1.1	1.1	1.1	1.1
47	0.5	0.5	0.5	0.5
48	0.5	0.5	0.5	0.5
49	1.1	1.1	1.1	1.1
50	8.5	8.5	2.2	2.2
51	1.1	1.1	1.1	1.1
52	1.1	1.1	1.1	1.1
53	1.1	1.1	0.5	0.5
54	1.1	1.1	0.3	0.3
55	4.3	4.3	2.2	2.2
56	1.1	2.1	0.5	0.5
57	4.3	4.3	2.2	2.2
58	0.5	0.5	0.5	0.5
59	4.3	4.3	0.5	0.5
60	0.5	0.5	0.3	0.3
61	0.5	0.5	0.5	0.5

Kaffir lime leaf oil

MIC range: 0.3-8.5 mg/ml

MIC_{50} = 1.1 mg/ml

MIC_{90} = 4.3 mg/ml

MBC range: 0.3-8.5 mg/ml

Kaffir lime oil

MIC range: 0.3-4.4 mg/ml

MIC_{50} = 1.1 mg/ml

MIC_{90} = 2.2 mg/ml

MBC range: 0.3-4.4 mg/ml

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Table 4.33 Disk diffusion of kaffir lime leaf oil and kaffir lime oil to Group B streptococci (27 isolates)

Lab No.	Kaffir lime leaf oil (mm)	Kaffir lime oil (mm)
1	11	11
2	14	11
3	14	12
4	15	11
5	14	12
6	14	12
7	13	10
8	17	12
9	14	11
10	14	12
11	12	10
12	14	11
13	14	11
14	14	12
15	17	13
16	13	11
17	14	12
18	15	11
19	15	12
20	14	12
21	15	12
22	14	11
23	13	11
24	16	11
25	14	12
26	15	11
27	14	11
Mean±S.D.	14.19±1.27	11.41±0.69

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Table 4.34 MIC and MBC values of kaffir lime leaf oil and kaffir lime oil to Group B streptococci (27 isolates)

Lab No.	Kaffir lime leaf oil (mg/ml)		Kaffir lime oil (mg/ml)	
	MIC	MBC	MIC	MBC
1	4.3	4.3	4.4	4.4
2	8.5	8.5	4.4	4.4
3	2.1	2.1	1.1	1.1
4	1.1	1.1	1.1	1.1
5	1.1	1.1	1.1	1.1
6	0.5	0.5	1.1	1.1
7	0.5	0.5	0.5	0.5
8	0.5	0.5	0.5	0.5
9	0.3	0.5	1.1	1.1
10	0.3	0.3	0.3	0.3
11	0.3	0.5	0.5	1.1
12	0.3	0.5	0.5	0.5
13	1.1	1.1	1.1	1.1
14	1.1	1.1	1.1	2.2
15	0.5	0.5	2.2	2.2
16	0.5	0.5	1.1	1.1
17	0.5	1.1	1.1	1.1
18	0.5	0.5	2.2	2.2
19	0.5	0.5	1.1	1.1
20	1.1	1.1	8.7	17.4
21	1.1	1.1	1.1	1.1
22	0.5	0.5	0.5	0.5
23	0.3	0.3	0.3	0.3
24	0.5	0.5	1.1	1.1
25	1.1	1.1	0.3	0.3
26	1.1	1.1	1.1	1.1
27	0.5	0.5	2.2	2.2

Kaffir lime leaf oil

MIC range: 0.3-8.5 mg/ml

MIC_{50} = 0.5 mg/ml

MIC_{90} = 1.1 mg/ml

MBC = range: 0.3-8.5 mg/ml

Kaffir lime oil

MIC range: 0.3-8.7 mg/ml

MIC_{50} = 1.1 mg/ml

MIC_{90} = 2.2 mg/ml

MBC range: 0.3-17.4 mg/ml

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Table 4.35 Disk diffusion of kaffir lime leaf oil and kaffir lime oil to Group C streptococci (4 isolates)

Lab No.	Kaffir lime leaf oil (mm)	Kaffir lime oil (mm)
1	15	12
2	15	13
3	14	12
4	14	12
Mean±S.D.	14.5±0.58	12.25±0.5

Table 4.36 MIC and MBC values of kaffir lime leaf oil and kaffir lime oil to Group C streptococci (4 isolates)

Lab No.	Kaffir lime leaf oil (mg/ml)		Kaffir lime oil (mg/ml)	
	MIC	MBC	MIC	MBC
1	4.3	4.3	4.4	4.4
2	2.1	4.3	4.4	4.4
3	0.3	0.3	0.3	0.3
4	0.5	0.5	0.5	1.1

Kaffir lime leaf oil

MIC range: 0.3-0.5 mg/ml

MIC_{50} = 0.6 mg/ml

MIC_{90} = 0.5 mg/ml

MBC range: 0.3-0.5 mg/ml

Kaffir lime oil

MIC range: 0.3-4.4 mg/ml

MIC_{50} = 0.5 mg/ml

MIC_{90} = 4.4 mg/ml

MBC range: 0.3-4.4 mg/ml

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Table 4.37 Disk diffusion of kaffir lime leaf oil and kaffir lime oil to Group F streptococci (3 isolates)

Lab No.	Kaffir lime leaf oil (mm)	Kaffir lime oil (mm)
1	18	15
2	15	18
3	20	16
Mean±S.D.	17.67±2.52	16.33±1.53

Table 4.38 MIC and MBC values of kaffir lime leaf oil and kaffir lime oil to Group F streptococci (3 isolates)

Lab No.	Kaffir lime leaf oil (mg/ml)		Kaffir lime oil (mg/ml)	
	MIC	MBC	MIC	MBC
1	2.1	2.1	1.1	1.1
2	4.3	4.3	4.4	4.4
3	0.3	0.3	0.5	0.5

Kaffir lime leaf oil

MIC range: 0.3-4.3 mg/ml

MIC_{50} = 2.1 mg/ml

MIC_{90} = 4.3 mg/ml

MBC range: 0.3-4.3 mg/ml

Kaffir lime oil

MIC range: 0.5-4.4 mg/ml

MIC_{50} = 1.1 mg/ml

MIC_{90} = 4.4 mg/ml

MBC range: 0.5-4.4 mg/ml

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Table 4.39 Disk diffusion of kaffir lime leaf oil and kaffir lime oil to Group G streptococci (11 isolates)

Lab No.	Kaffir lime leaf oil (mm)	Kaffir lime oil (mm)
1	16	13
2	15	13
3	16	14
4	16	13
5	15	12
6	16	12
7	16	14
8	16	15
9	17	13
10	15	12
11	14	12
Mean±S.D.	15.64±0.81	13.0±1

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Table 4.40 MIC and MBC values of kaffir lime leaf oil and kaffir lime oil to Group G streptococci (11 isolates)

Lab No.	Kaffir lime leaf oil (mg/ml)		Kaffir lime oil (mg/ml)	
	MIC	MBC	MIC	MBC
1	4.3	4.3	4.4	4.4
2	2.1	2.1	1.1	2.2
3	2.1	4.3	4.4	4.4
4	0.3	0.3	0.3	0.5
5	0.5	0.5	0.3	0.5
6	0.3	0.5	1.1	1.1
7	0.3	0.5	0.5	0.5
8	0.5	1.1	1.1	1.1
9	2.1	4.3	1.1	2.2
10	0.3	0.3	0.5	0.5
11	1.1	2.1	1.1	1.1

Kaffir lime leaf oil

MIC range: 0.3-4.3 mg/ml

MIC_{50} = 0.3 mg/ml

MIC_{90} = 2.1 mg/ml

MBC range: 0.3-4.3 mg/ml

Kaffir lime oil

MIC range: 0.3-4.4 mg/ml

MIC_{50} = 1.1 mg/ml

MIC_{90} = 4.4 mg/ml

MBC range: 0.5-4.4 mg/ml

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Table 4.41 Disk diffusion of kaffir lime leaf oil and kaffir lime oil to *S. pneumoniae* (51isolates)

Lab No.	Kaffir lime leaf oil (mm)	Kaffir lime oil (mm)
1	24	20
2	25	17
3	23	18
4	15	12
5	22	17
6	29	19
7	26	20
8	20	16
9	27	17
10	27	20
11	25	17
12	26	17
13	38	22
14	19	15
15	20	14
16	20	16
17	26	15
18	23	19
19	35	19
20	21	19
21	27	20
22	22	18
23	30	19
24	25	15
25	21	18
26	27	17
27	35	17
28	25	17
29	33	16
30	23	19
31	25	19
32	27	20
33	25	16
34	33	16
35	22	14

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Table 4.41 Disk diffusion of kaffir lime leaf oil and kaffir lime oil to *S. pneumoniae* (51isolates)

Lab No.	Kaffir lime leaf oil (mm)	Kaffir lime oil (mm)
36	23	16
37	22	18
38	35	19
39	22	18
40	23	16
41	30	18
42	18	15
43	30	16
44	21	15
45	22	16
46	22	16
47	22	13
48	23	18
49	26	16
50	19	14
51	22	17
Mean±S.D.	24.92±4.82	17.08±2.04

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Table 4.42 MIC and MBC values of kaffir lime leaf oil and kaffir lime oil to *S. pneumoniae* (51isolates)

Lab No.	Kaffir lime leaf oil (mg/ml)		Kaffir lime oil (mg/ml)	
	MIC	MBC	MIC	MBC
1	0.3	0.3	0.3	0.3
2	1.1	2.1	0.5	0.5
3	1.1	1.1	1.1	1.1
4	0.3	1.1	0.3	0.5
5	0.3	0.5	0.3	1.1
6	1.1	1.1	1.1	1.1
7	0.5	1.1	2.2	2.2
8	1.1	1.1	1.1	1.1
9	1.1	1.1	1.1	1.1
10	0.3	0.3	0.5	0.5
11	0.3	1.1	0.5	1.1
12	0.5	0.5	1.1	1.1
13	0.5	0.5	0.5	0.5
14	0.5	1.1	0.5	2.2
15	1.1	1.1	1.1	1.1
16	0.3	1.1	0.5	0.5
17	4.3	4.3	4.4	4.4
18	1.1	1.1	1.1	1.1
19	0.5	0.5	0.5	0.5
20	2.1	2.1	0.5	2.2
21	0.5	1.1	1.1	2.2
22	0.5	1.1	0.5	2.2
23	0.5	0.5	1.1	1.1
24	1.1	4.3	1.1	4.4
25	0.3	1.1	0.3	0.3
26	0.3	0.5	0.5	0.5
27	0.3	0.5	0.5	0.5
28	0.5	1.1	0.5	0.5
29	2.1	2.1	1.1	1.1
30	0.3	0.3	1.1	1.1
31	0.5	1.1	2.2	1.1
32	2.1	2.1	1.1	1.1
33	1.1	1.1	0.5	2.2

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Table 4.42 MIC and MBC values of kaffir lime leaf oil and kaffir lime oil to *S. pneumoniae* (51isolates)

Lab No.	Kaffir lime leaf oil (mg/ml)		Kaffir lime oil (mg/ml)	
	MIC	MBC	MIC	MBC
34	0.5	0.5	0.5	0.5
35	1.1	2.1	1.1	1.1
36	0.5	1.1	1.1	1.1
37	1.1	1.1	1.1	1.1
38	0.5	4.3	1.1	1.1
39	4.3	4.3	2.2	4.4
40	2.1	8.5	2.2	4.4
41	0.3	0.5	0.5	0.5
42	0.3	2.1	1.1	1.1
43	0.5	0.5	0.5	0.5
44	0.3	0.5	0.5	0.5
45	0.5	1.1	1.1	1.1
46	0.5	0.5	0.5	0.5
47	0.3	2.1	1.1	1.1
48	1.1	2.1	0.5	2.2
49	0.3	0.5	0.5	0.5
50	0.3	0.5	0.5	0.5
51	1.1	4.3	0.5	1.1

Kaffir lime leaf oil

MIC range: 0.3-4.3 mg/ml

MIC_{50} = 0.5 mg/ml

MIC_{90} = 2.1 mg/ml

MBC range: 0.3-8.5 mg/ml

Kaffir lime oil

MIC range: 0.3-4.4 mg/ml

MIC_{50} = 0.5 mg/ml

MIC_{90} = 1.1 mg/ml

MBC range: 0.3-4.4 mg/ml

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Table 4.43 Disk diffusion of kaffir lime leaf oil and kaffir lime oil to MRSA (50 isolates)

Lab No.	Kaffir lime leaf oil (mm)	Kaffir lime oil (mm)
1	15	20
2	11	20
3	10	20
4	12	25
5	12	23
6	13	26
7	12	25
8	11	25
9	11	25
10	13	26
11	10	22
12	10	25
13	13	24
14	13	25
15	12	19
16	12	24
17	14	25
18	15	25
19	13	22
20	14	25
21	14	22
22	16	23
23	13	24
24	12	25
25	12	24
26	13	21
27	13	25
28	15	22
29	15	23
30	13	25
31	12	19
32	12	22
33	12	25
34	10	21
35	12	23
36	12	23

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Table 4.43 Disk diffusion of kaffir lime leaf oil and kaffir lime oil to MRSA (50 isolates)

Lab No.	Kaffir lime leaf oil (mm)	Kaffir lime oil (mm)
37	13	26
38	11	20
39	11	21
40	13	25
41	13	25
42	13	23
43	12	24
44	12	22
45	13	24
46	14	22
47	14	23
48	13	25
49	15	25
50	14	24
Mean±S.D.	12.66±1.42	23.34±1.93

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Table 4.44 MIC and MBC values of kaffir lime leaf oil and kaffir lime oil to MRSA (50 isolates)

Lab No.	Kaffir lime leaf oil (mg/ml)		Kaffir lime oil (mg/ml)	
	MIC	MBC	MIC	MBC
1	8.5	34	2.2	4.4
2	8.5	34	4.4	8.7
3	17	34	4.4	4.4
4	8.5	34	4.4	4.4
5	17	34	17.4	8.7
6	17	34	8.7	8.7
7	17	34	4.4	8.7
8	8.5	68	4.4	4.4
9	68	68	2.2	8.7
10	4.3	34	2.2	4.4
11	17	68	4.4	8.7
12	8.5	17	4.4	8.7
13	8.5	34	2.2	4.4
14	4.3	34	2.2	8.7
15	4.3	34	2.2	2.2
16	4.3	34	2.2	4.4
17	8.5	34	2.2	8.7
18	4.3	34	2.2	8.7
19	8.5	34	2.2	8.7
20	4.3	17	2.2	4.4
21	34	68	2.2	2.2
22	8.5	34	2.2	8.7
23	8.5	34	2.2	4.4
24	4.3	17	2.2	4.4
25	8.5	34	2.2	8.7
26	8.5	34	2.2	4.4
27	8.5	17	2.2	4.4
28	8.5	17	4.4	4.4
29	4.3	8.5	2.2	2.2
30	2.1	17	2.2	4.4
31	4.3	34	2.2	4.4
32	8.5	8.5	2.2	8.7
33	8.5	8.5	4.4	4.4
34	17	34	2.2	2.2

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Table 4.44 MIC and MBC values of kaffir lime leaf oil and kaffir lime oil to MRSA (50 isolates)

Lab No.	leaf oil (mg/ml)		Kaffir lime oil (mg/ml)	
	MIC	MBC	MIC	MBC
35	17	17	2.2	2.2
36	8.5	8.5	2.2	4.4
37	34	34	2.2	2.2
38	34	34	4.4	4.4
39	68	68	2.2	4.4
40	34	34	2.2	4.4
41	2.1	34	1.1	2.2
42	17	34	1.1	4.4
43	8.5	68	1.1	17.4
44	4.3	17	2.2	4.4
45	2.1	68	2.2	4.4
46	4.3	8.5	2.2	2.2
47	34	34	4.4	4.4
48	4.3	17	2.2	2.2
49	8.5	8.5	2.2	2.2
50	4.3	8.5	2.2	2.2

Kaffir lime leaf oil

MIC range: 2.1-68 mg/ml

MIC_{50} = 8.5 mg/ml

MIC_{90} = 34 mg/ml

MBC range: 8.5-68 mg/ml

Kaffir lime oil

MIC range: 1.1-17.4 mg/ml

MIC_{50} = 2.2 mg/ml

MIC_{90} = 4.4 mg/ml

MBC range: 2.2-17.4 mg/ml

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Table 4.45 Disk diffusion of kaffir lime leaf oil and kaffir lime oil to MSSA (50 isolates)

Lab No.	Kaffir lime leaf oil (mm)	Kaffir lime oil (mm)
1	14	21
2	12	22
3	12	23
4	13	22
5	11	23
6	11	22
7	12	24
8	10	22
9	10	22
10	12	22
11	10	21
12	11	23
13	13	22
14	11	23
15	12	23
16	13	21
17	11	25
18	10	23
19	11	24
20	12	25
21	12	24
22	12	22
23	12	18
24	13	22
25	13	24
26	12	25
27	11	21
28	13	21
29	12	25
30	11	23
31	14	25
32	10	21
33	12	21
34	10	22
35	11	22

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Table 4.45 Disk diffusion of kaffir lime leaf oil and kaffir lime oil to MSSA (50 isolates)

Lab No.	Kaffir lime leaf oil (mm)	Kaffir lime oil (mm)
36	11	20
37	12	23
38	11	23
39	12	24
40	13	26
41	13	24
42	12	23
43	13	21
44	11	23
45	12	24
46	13	25
47	12	23
48	14	23
49	13	23
50	11	19
Mean±S.D.	11.84±1.09	22.66±1.61

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Table 4.46 MIC and MBC values of kaffir lime leaf oil and kaffir lime oil to MSSA (50 isolates)

Lab No.	Kaffir lime leaf oil (mg/ml)		Kaffir lime oil (mg/ml)	
	MIC	MBC	MIC	MBC
1	4.3	34	2.2	4.4
2	34	68	8.7	8.7
3	8.5	34	4.4	4.4
4	8.5	34	2.2	4.4
5	8.5	34	4.4	4.4
6	17	34	8.7	8.7
7	8.5	34	2.2	4.4
8	8.5	34	2.2	4.4
9	17	17	4.4	4.4
10	4.3	17	2.2	4.4
11	8.5	17	2.2	2.2
12	8.5	8.5	1.1	2.2
13	2.1	17	2.2	2.2
14	17	17	1.1	2.2
15	4.3	34	4.4	8.7
16	2.1	34	2.2	4.4
17	4.3	34	2.2	4.4
18	4.3	34	2.2	2.2
19	2.1	17	1.1	2.2
20	4.3	17	2.2	4.4
21	8.5	34	2.2	4.4
22	8.5	34	4.4	4.4
23	17	34	2.2	8.7
24	8.5	34	2.2	4.4
25	4.3	17	2.2	4.4
26	17	68	2.2	4.4
27	17	34	4.4	8.7
28	8.5	8.5	2.2	4.4
29	8.5	34	4.4	8.7
30	17	17	4.4	8.7
31	17	34	2.2	2.2
32	17	34	1.1	2.2
33	8.5	8.5	1.1	2.2
34	17	17	2.2	4.4

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Table 4.46 MIC and MBC values of kaffir lime leaf oil and kaffir lime oil to MSSA (50 isolates)

Lab No.	Kaffir lime leaf oil (mg/ml)		Kaffir lime oil (mg/ml)	
	MIC	MBC	MIC	MBC
35	34	68	2.2	2.2
36	34	34	2.2	4.4
37	17	34	1.1	4.4
38	17	34	2.2	4.4
39	2.1	34	4.4	8.7
40	1.1	17	2.2	4.4
41	4.3	68	1.1	4.4
42	2.1	17	2.2	4.4
43	4.3	34	2.2	8.7
44	8.5	34	2.2	4.4
45	8.5	34	1.1	4.4
46	8.5	17	1.1	4.4
47	4.3	34	2.2	4.4
48	4.3	34	1.1	4.4
49	4.3	34	2.2	4.4
50	8.5	34	2.2	4.4

Kaffir lime leaf oil

MIC range: 1.1-34 mg/ml

MIC_{50} = 8.5 mg/ml

MIC_{90} = 17 mg/ml

MBC range: 8.5-68 mg/ml

Kaffir lime oil

MIC range: 1.1-8.7 mg/ml

MIC_{50} = 2.2 mg/ml

MIC_{90} = 4.4 mg/ml

MBC range: 2.2-8.7 mg/ml

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Table 4.47 Disk diffusion of kaffir lime leaf oil and kaffir lime oil to *A. baumannii* (50 isolates)

Lab No.	Kaffir lime leaf oil (mm)	Kaffir lime oil (mm)
1	10	22
2	9	20
3	10	21
4	10	22
5	9	20
6	9	21
7	9	22
8	10	20
9	10	20
10	11	22
11	9	21
12	10	24
13	9	20
14	12	21
15	8	20
16	9	22
17	9	20
18	10	22
19	9	24
20	11	23
21	11	22
22	9	21
23	10	22
24	9	20
25	10	21
26	8	22
27	10	25
28	9	23
29	10	24
30	9	22
31	8	20
32	9	22
33	9	23
34	9	23
35	8	24

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Table 4.47 Disk diffusion of kaffir lime leaf oil and kaffir lime oil to *A. baumannii* (50 isolates)

Lab No.	Kaffir lime leaf oil (mm)	Kaffir lime oil (mm)
36	8	22
37	9	25
38	9	25
39	9	21
40	9	20
41	9	25
42	8	20
43	9	22
44	11	25
45	8	20
46	9	23
47	9	24
48	9	21
49	9	23
50	9	22
Mean±S.D.	9.22±1.07	21.98±1.61

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Table 4.48 MIC and MBC values of kaffir lime leaf oil and kaffir lime oil to *A. baumannii* (50 isolates)

Lab No.	Kaffir lime leaf oil (mg/ml)		Kaffir lime oil (mg/ml)	
	MIC	MBC	MIC	MBC
1	8.5	0.85	1.1	1.1
2	4.3	8.5	4.4	4.4
3	2.1	8.5	2.2	2.2
4	4.3	4.3	4.4	4.4
5	4.3	4.3	1.1	1.1
6	4.3	4.3	1.1	1.1
7	8.5	8.5	1.1	1.1
8	4.3	4.3	1.1	4.4
9	17	34	1.1	1.1
10	2.1	34	1.1	4.4
11	8.5	17	1.1	2.2
12	8.5	17	1.1	2.2
13	17	17	1.1	2.2
14	17	1.7	1.1	4.4
15	4.3	8.5	1.1	1.1
16	8.5	17	1.1	2.2
17	8.5	34	1.1	4.4
18	4.3	17	1.1	2.2
19	8.5	34	1.1	2.2
20	4.3	34	1.1	4.4
21	4.3	8.5	1.1	2.2
22	8.5	34	1.1	4.4
23	8.5	68	1.1	4.4
24	4.3	17	1.1	1.1
25	4.3	8.5	1.1	1.1
26	4.3	8.5	4.4	4.4
27	2.1	17	1.1	4.4
28	4.3	34	1.1	2.2
29	2.1	8.5	1.1	4.4
30	4.3	17	1.1	8.7
31	4.3	34	1.1	2.2
32	4.3	17	1.1	4.4
33	4.3	17	2.2	4.4
34	4.3	8.5	2.2	2.2

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Table 4.48 MIC and MBC values of kaffir lime leaf oil and kaffir lime oil to *A. baumannii* (50 isolates)

Lab No.	Kaffir lime leaf oil (mg/ml)		Kaffir lime oil (mg/ml)	
	MIC	MBC	MIC	MBC
35	8.5	17	1.1	4.4
36	8.5	34	1.1	4.4
37	4.3	17	1.1	8.7
38	8.5	17	4.4	4.4
39	8.5	34	2.2	4.4
40	4.3	8.5	2.2	4.4
41	8.5	17	1.1	8.7
42	8.5	8.5	2.2	2.2
43	8.5	34	1.1	2.2
44	4.3	17	4.4	4.4
45	17	8.5	4.4	4.4
46	8.5	17	1.1	1.1
47	17	34	1.1	2.2
48	8.5	34	1.1	4.4
49	8.5	17	1.1	8.7
50	8.5	17	1.1	4.4

Kaffir lime leaf oil

MIC range: 2.1-17 mg/ml

MIC_{50} = 4.3 mg/ml

MIC_{90} = 8.5 mg/ml

MBC range: 4.3-68 mg/ml

Kaffir lime oil

MIC range: 1.1-4.4 mg/ml

MIC_{50} = 1.1 mg/ml

MIC_{90} = 4.4 mg/ml

MBC: 1.1-8.7 mg/ml

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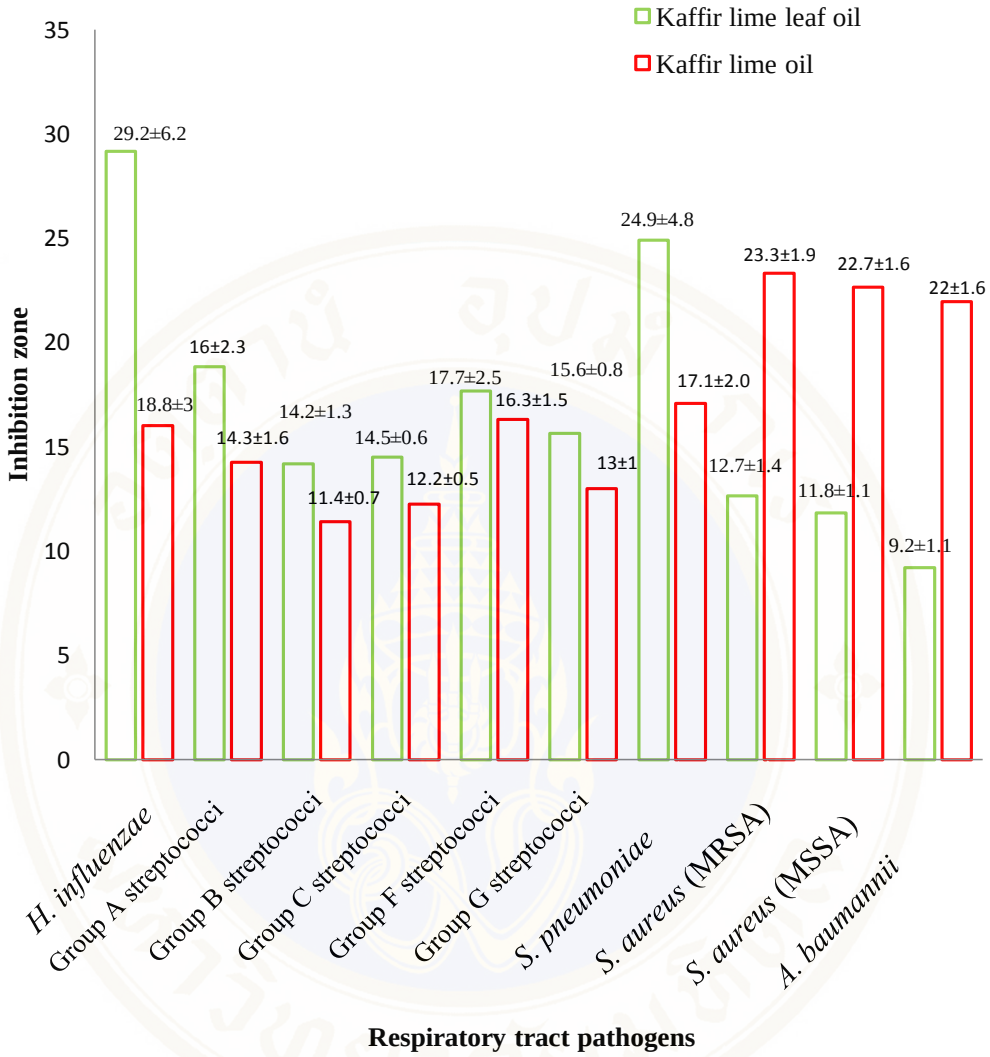

Numbers represent mean ± standard deviation of inhibition zone (mm).

Figure 4.35 Inhibition zones of kaffir lime leaf oil and kaffir lime oil for various respiratory tract pathogens by disk diffusion method

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Table 4.49 MIC and MBC values of kaffir lime leaf oil for bacterial pathogens

Pathogens	**Kaffir lime leaf oil (mg/ml)**			
	MIC range	MIC_{50}	MIC_{90}	MBC range
H. influenzae	0.06-0.5	0.3	0.3	0.06-1.1
Group A streptococci	0.3-8.5	1.1	4.3	0.3-8.5
Group B streptococci	0.3-8.5	0.5	1.1	0.3-8.5
Group C streptococci	0.3-4.3	0.5	4.3	0.3-4.3
Group F streptococci	0.3-4.3	2.1	4.3	0.3-4.3
Group G streptococci	0.3-4.3	0.3	2.1	0.3-4.3
S. pneumoniae	0.3-4.3	0.5	2.1	0.3-8.5
S. aureus (MRSA)	2.1-68	8.5	34	8.5-68
S. aureus (MSSA)	1.1-34	8.5	17	8.5-68
A. baumannii	2.1-17	4.3	8.5	4.3-68

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Table 4.50 MIC and MBC values of kaffir lime oil for bacterial pathogens

Pathogens	**Kaffir lime oil (mg/ml)**			
	MIC range	MIC_{50}	MIC_{90}	MBC range
H. influenzae	0.06-0.5	0.1	0.3	0.06-1.1
Group A streptococci	0.3-4.4	1.1	2.2	0.3-4.4
Group B streptococci	0.3-8.7	1.1	2.2	0.3-17.4
Group C streptococci	0.3-4.4	0.5	4.4	0.3-4.4
Group F streptococci	0.5-4.4	1.1	4.4	0.5-4.4
Group G streptococci	0.3-4.4	1.1	4.4	0.5-4.4
*S. pneumonia*e	0.3-4.4	0.5	1.1	0.3-4.4
S. aureus (MRSA)	1.1-17.4	2.2	4.4	2.2-17.4
S. aureus (MSSA)	1.1-8.7	2.2	4.4	2.2-8.7
A. baumannii	1.1-4.4	1.1	4.4	1.1-8.7

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Table 4.51 Effect of citronellal in kaffir lime leaf oil on bacterial pathogens

Pathogens	Citronellal			Kaffir lime leaf oil		
	Inhibition zone (mm)	MIC (mg/ml)	MBC (mg/ml)	Inhibition zone (mm)	MIC (mg/ml)	MBC (mg/ml)
H. influenzae	20	0.5	0.5	21	0.3	0.3
Group A streptococci	15	0.5	1.1	16	4.3	4.3
Group B streptococci	10	1.1	1.1	11	4.3	4.3
Group C streptococci	15	0.3	0.5	15	4.3	4.3
Group F streptococci	17	1.1	1.1	17	2.1	8.5
Group G streptococci	15	1.1	1.1	16	4.3	4.3
S. pneumoniae	24	0.5	0.5	24	0.3	0.3
S. aureus (MRSA)	15	1.1	8.57	15	8.5	34
S. aureus (MSSA)	14	1.1	8.57	14	4.3	34
A. baumannii	13	1.1	4.3	10	8.5	8.5
H. influenzae ATCC 49766	20	0.3	0.5	22	0.5	0.5
*S. pneumonia*e ATCC 49619	23	0.5	0.5	23	0.5	0.5
S. aureus ATCC 29213	22	1.1	1.1	19	1.1	1.1

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Table 4.52 Effect of limonene in kaffir lime oil on bacterial pathogens

Pathogens	**Limonene**			**Kaffir lime oil**		
	Inhibition zone (mm)	MIC (mg/ml)	MBC (mg/ml)	Inhibition zone (mm)	MIC (mg/ml)	MBC (mg/ml)
H. influenzae	12	67.2	67.2	17	0.3	0.3
Group A streptococci	6	>134.4	>134.4	14	2.2	2.2
Group B streptococci	7	>134.4	>134.4	11	4.4	4.4
Group C streptococci	6	>134.4	>134.4	12	4.4	4.4
Group F streptococci	6	>134.4	>134.4	15	1.1	1.1
Group G streptococci	6	>134.4	>134.4	13	4.4	4.4
S. pneumoniae	11	33.6	67.2	20	0.3	0.3
S. aureus (MRSA)	6	>134.4	>134.4	20	2.2	2.2
S. aureus (MSSA)	6	>134.4	>134.4	21	4.4	4.4
A. baumannii	6	>134.4	>134.4	22	1.1	1.1
H. influenzae ATCC 49766	12	67.2	67.2	17	0.1	0.1
S. pneumoniae ATCC 49619	8	67.2	>134.4	16	0.5	0.5
S. aureus ATCC 29213	11	>134.4	>134.4	26	1.1	1.1

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Table 4.53 Effect of alpha-terpineol in kaffir lime oil on bacterial pathogens

Pathogens	**Alpha-terpineol**		**Kaffir lime oil**	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
H. influenzae	0.07	0.07	0.3	0.3
Group A streptococci	0.6	0.6	2.2	2.2
Group B streptococci	0.6	0.6	4.4	4.4
Group C streptococci	0.6	0.6	4.4	4.4
Group F streptococci	1.2	1.2	1.1	1.1
Group G streptococci	1.2	1.2	4.4	4.4
S. pneumoniae	0.3	0.3	0.3	0.3
S. aureus (MRSA)	2.4	2.4	2.2	2.2
S. aureus (MSSA)	2.4	2.4	4.4	4.4
A. baumannii	0.07	0.1	1.1	1.1
H. influenzae ATCC 49766	0.07	0.07	0.1	0.1
S. pneumoniae ATCC 49619	0.6	0.6	0.5	0.5
S. aureus ATCC 29213	0.3	1.2	1.1	1.1

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Table 4.54 MIC and MBC values for 12% kaffir lime 1eaf oil at room temperature

MIC and MBC of 12% kaffir lime leaf oil (mg/ml)							
Group A streptococci		*S. pneumoniae* 49619		*S. aureus* 29213		*H. influenzae* 49766	
MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1 month							
23.2	23.2	11.6	11.6	92.8	92.8	1.45	2.9
2 months							
23.2	23.2	11.6	11.6	46.4	185.6	2.9	5.8
3 months							
23.2	23.2	5.8	11.6	46.4	185.6	1.45	1.45
4 months							
23.2	23.2	2.9	2.9	46.4	46.4	1.45	2.9

Table 4.55 MIC and MBC values for 10% kaffir lime oil at room temperature

MIC and MBC of 10% kaffir lime oil (mg/ml)							
Group A streptococci		*S. pneumoniae* 49619		*S. aureus* 29213		*H. influenzae* 49766	
MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1 month							
42.4	42.4	10.6	10.6	84.8	84.8	1.7	1.7
2 months							
21.2	21.2	10.6	21.2	42.4	169.6	5.3	5.3
3 months							
21.2	21.2	5.3	21.2	42.4	169.6	2.7	2.7
4 months							
21.2	42.4	5.3	5.3	42.4	84.8	5.3	5.3

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CHAPTER V
DISCUSSION

Although *H. influenzae* is usually sensitive to beta-lactams (20), the increasing prevalence of resistant isolates occurs (209-211) and leads to a serious issue relating to the appropriate antibiotic selection for treatment of respiratory tract infection worldwide. Therefore, it is important to monitor antimicrobial activity and the resistant mechanisms for obtaining optimal therapy. Susceptibility to all beta-lactams in *H. influenzae* is mostly predicted by susceptibility to ampicillin according to CLSI (8). The variation of ampicillin resistance in this organism is 10-60% which depends on geographical region. The resistant mechanisms are mediated by the presence of beta-lactamase, mutations in transpeptidase domain of PBP (BLNAR isolates) (1, 34, 212, 213), or the presence of both beta-lactamase and mutations in PBP (BLPACR) isolates (4, 5, 213, 214).

In this study, 100 beta-lactamase producing *H. influenzae* isolates collected between 2009-2010 were tested against 9 antimicrobial agents. All of isolates were resistant to ampicillin. They were 100% susceptible to third generation cephalosporins; cefotaxime, ceftriaxone and also 100% susceptible to ciprofloxacin while ceftazidime, a one kind of third generation cephalosporin, was less susceptible with 67%. Improper use of ceftazidime may exert selective pressure on resistance for this drug (215). As tetracycline with 47% and trimethoprim-sulphamethoxazole with 28% susceptibility were less susceptible in these tested isolates. This result correlated with the previous study in Poland, which resistance to tetracycline was found more often among BLPAR isolates (22.6%) than among BLNAS isolates (0.7%) or among BLNAR isolates (2.2%) (215), although both tetracycline and sulfa drugs remain effective in treatment of sinusitis or respiratory infection (216). While amoxicillin/clavulanic acid, a combined drug of beta-lactams and beta-lactamase inhibitor, is one of drugs of choice, which can inhibit activity of beta-lactamase.

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Amoxicillin/clavulanic acid did not show 100% susceptibility, it showed only 88%, which this phenotype may involve (i) hyperproduction of TEM-1 or ROB-1 beta-lactamase, (ii) production of an altered TEM-1 or ROB-1 beta-lactamase that become resistant to clavulanate inhibition, (iii) production of a novel beta-lactamase that is not effectively inhibited by clavulanate, (iv) possess both mechanisms of beta-lactamase and changes in PBP (BLPACR) isolates or (v) efflux mechanisms. Although previous studies demonstrated that efflux systems and lowered cell permeability were not mechanisms of resistance to beta-lactams in *H. influenzae* (119, 177, 217), there was a report of efflux activity which resulted in increasing the resistance level but due originally to altered PBP (212).

All 26 BLNAR isolates in this study were resistant to ampicillin. They were susceptible to ciprofloxacin (100%), cefotaxime, ceftriaxone, ceftazidime (88.46% each), amoxicillin/clavulanic acid (80.77%) and cefuroxime (42.31%). Therefore, BLNAR isolates should be considered resistant to amoxicillin-clavulanic acid, ampicillin-sulbactam, cefaclor, cefetamet, cefonicid, cefprozil, cefuroxime, and loracarbef according to CLSI (8). Ampicillin was usually used to treat patients infected with *H. influenzae* isolates that negative to beta-lactamase. While now increasing in the prevalence of these isolates in many countries may result from the prescription of antibiotics. In this result, BLNAR isolates showed more resist to beta-lactams than beta-lactamase producing isolates. Therefore, the misuse or various third generation cephalosporin usages may increase this resistant isolates and reduce susceptibility to various kind of third generation cephalosporins. For tetracycline and trimethoprim-sulphamethoxazole, BLNAR isolates showed 57.69% and 26.92% susceptibility, respectively. This result correlated with the susceptibility in beta-lactamase producing isolates.

In *H. influenzae*, the production of beta-lactamase is the most common mechanism of beta-lactam resistance (1). Study in 1999-2000 from one international surveillance in 2,948 isolates showed 16.6% of beta-lactamase producing isolates and 0.07% of BLNAR isolates (5). Beta-lactamase producing isolates ranged from 3% in Germany to 65% in South Korea (5). In addition, TEM-1 type was predominated in beta-lactamase producing isolates compared to ROB-1 type (148, 209). Prevalence of

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ROB-1 among beta-lactamase producing isolates may vary according to geographic location. The highest rate of ROB-1 type was found in Mexico (30%), followed by USA (13.2%) and Canada (9.4%) (13). In this study, beta-lactamase producing *H. influenzae* isolates indicated 99% was TEM type and 1% was ROB-1 type. This result was in accordance with other study which prevalence of 93.7% was TEM-1, 4.6% was ROB-1, 1.2% was neither gene, 0.04% was both genes in overall 2,225 beta-lactamase producing isolates of global distribution of beta-lactamases in *H. influenzae* from PROTEKT (Prospective Resistant Organism Tracking and study Epidemiology for the Ketolide Telithromycin) study during 1999–2003 (5). There was 7% of 157 beta-lactamase producing isolates containing ROB-1 type and 93% containing TEM in Canada during 1992 and 1993 (148). The nationwide acute respiratory infection surveillance (ARIS) network in Korea during 2005 and 2006 reported 94.7% and 3.2% in TEM-1 and ROB-1 types, respectively of *H. influenzae* isolates (218).

The prevalence of beta-lactamase producing isolates in some countries decreased e.g., in Spain decreased from 28% in 1998 to 16% in 2000, the USA decreased from 36% in 1994 to 26% in 2002 and Japan decreased from 25% in 1995 to 3% in 1999 (15, 214, 219). None of *H. influenzae* contained both in TEM and ROB-1 type. This result correlated with the previous study which isolates containing both TEM-1 and ROB-1 type were rare and found in only one report (13). In addition, the supported data on the nature of plasmids in these rare isolates of *H. influenzae* that express both TEM-1 and ROB-1 is unknown, although report of 50 isolates *H. ducreyi* in Thailand were found to carry two small plasmids that individually carried TEM-1 and ROB-1 genes (164).

Prevalence of BLNAR isolates is still low worldwide e.g., 2% in Australia, study in over 1,000 isolates from SENTRY surveillance programme during 1998-2003 (221). This data related to global prevalence in Alexander Project from 8,523 isolates of *H. influenzae* during 1998-2000 from 26 countries which found 0.2% BLNAR isolates (219) and prevalence in USA study where 0.8% and 2.5% found in isolates with ampicillin MIC of ≥4 µg/ml and MIC of ≥2 µg/ml (222). The low prevalence of BLNAR isolates may be due to methodological problems e.g., lack of a general agreement in definition in breakpoint, technical performance (19) and lack of beta-

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lactamase test in some laboratories. However, there are some countries where BLNAR isolates are increasing e.g., Poland had the highest rate of BLNAR isolates (20%) from respiratory tract infections in Europe (4). In addition, there was the emergence of BLNAR isolates in many European countries ranging 2-20% (4) e.g., 33.8% were beta-lactamase producers (TEM type) and 18.9% were reduced susceptibility to beta-lactams via target modification (BLNAR isolates) in France (224).

Interestingly, Japan found prevalence of BLNAR isolates greatly increased. Previous report in Japan showed that BLNAR from Hib isolates increasing from 5.8% in 2000 to 34.5% in 2004 (225). Later study in four cities in Hokkaido, Japan in 2004 based on PCR genotyping found 44.2% BLNAS isolates. There were 10.4% low-BLNAR isolates, 39% high-BLNAR isolates, 2.6% BLPAR isolates and 3.9% BLPACR isolates (20). Later study was performed to compare prevalence of BLNAR isolates between October 2000-July 2001 and January-June 2004 which found the proportion of BLNAS isolates decreased from 62.9% to 34.3% while proportions of BLPAR isolates were 8.3% and 6.4% in these 2 periods of time but proportion of BLNAR isolates increased from 28.8% in the first period to 59.3% in the second period (226). During October 2005-March 2008, BLNAR isolates (49.2%) were isolated from middle ear fluid of pediatric acute otitis media patients (2). Therefore, the prevalence of BLNAR isolates in Japan is consistently higher rates than in Europe and USA. This situation may result from different prescribing characteristics (16, 22, 225). In Europe and USA, the first-line drug for treatment of community-acquired respiratory infections is amoxicillin/clavulanic acid whereas oral cephalosporins, especially cefdinir and cefditoren are widely used in Japan (22). Moreover, cefdinir and cefditoren are often used in low doses, therefore it may cause only partial microbial damage without bactericidal activity and may allow the emergence of mutant isolates (22). As in this study, an increase of BLNAR isolates in Thailand was shown although it has some variation in 2009 which the proportion decreased from 3.95% in 2008 to 3.32% in 2009, but the overall tested isolates increased from 3.95% in 2008 to 8.39% in 2010.

Susceptibility to ampicillin by broth microdilution method demonstrated that ampicillin-MIC of all beta-lactamase producing isolates ranged 8->512 µg/ml

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which approximately 80% is ≥128 µg/ml. The ampicillin-MIC of BLNAR isolates was found in range 2-16 µg/ml which approximately 87.5% is ≤4 µg/ml. This result was in accordance with the study in susceptibility of *H. influenzae* from institutions that participated in a nationwide drug-resistant pathogen surveillance group in pediatric infectious disease in Japan between 2 phases as previously mentioned (phase 1, October 2000-July 2001; phase 2, January-June 2004) which the MIC of beta-lactamase producing isolates were 2–128 µg/ml in phase 1 and 16–>128 µg/ml in phase 2 while MIC ranged 2–4 µg/ml in phase 1 and 2–32 µg/ml in phase 2 were found in BLNAR isolates (226). In addition, our result also in accordance with the study of 544 *H. influenzae* isolates through the nationwide acute respiratory infection surveillance (ARIS) network in Korea during 2005 and 2006 which MIC of beta-lactamase producing isolates were 2–≥512 µg/ml while 2–8 µg/ml were found in BLNAR isolates (218). Therefore, ampicillin-MIC of beta-lactamase producing isolates is higher than ampicillin-MIC of BLNAR isolates indicating *H. influenzae* that produced beta-lactamase has higher level of ampicillin resistance than BLNAR isolates (223). Amoxicillin/clavulanic acid-MIC values in BLNAR isolates found in range 0.75-4 µg/ml which still susceptible to this drug. Importantly, CLSI recommends that all BLNAR isolates should be reported as resistant to amoxicillin/clavulanic acid, ampicillin-sulbactam, cefaclor, cefetamet, cefonicid, cefprozil, cefuroxime, and loracarbef despite some of these isolates test in vitro still obviously susceptible to these antibiotics (8). In addition, BLNAR isolates in this study were categorized as high BLNAR because many studies suggested that high BLNAR isolates have ampicillin-MICs in range 1-16 µg/ml while low-BLNAR isolates have ampicillin MICs in range 0.5-2 µg/ml (16, 25-26). Therefore, our BLNAR isolates which were high-BLNAR can resist beta-lactams more than low-BLNAR isolates. Furthermore, previous studies of BLNAR isolates from Japan, France and North America reported that their ampicillin-MIC were in range 1-4 µg/ml (212) while 11.54% of our BLNAR isolates had ampicillin-MIC in range 8-16 µg/ml which is a high ampicillin-MIC in BLNAR isolates. From a study in North America, BLNAR isolates with high ampicillin-MIC may occur from combined resistance mechanisms in PBP3 and AcrAB efflux pump (212). They demonstrated that the

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regulation of AcrAB efflux can decrease the potency of ampicillin in combination with reduced PBP3 and lead to increased ampicillin-MIC levels in range 8-16 µg/ml (212). Although efflux pump mechanism can raise ampicillin-MIC levels, but only mutations in PBP3 cause this characteristic e.g., surveillance studies in USA found BLNAR isolates with ampicillin-MIC of 16 µg/ml, although they are rare cases (16). In Japan, BLNAR isolates with high MIC were also identified in recent studies (16). Therefore, investigation whether increased efflux of ampicillin by examination of AcrAB repressor gene or *acrR* mutations leading to higher ampicillin-MIC in BLNAR isolates compared with *H. influenzae* Rd isolate may be an important thing. This is because an increased mutation rate in several loci may lead to complication of treatment of respiratory tract infections with ampicillin or amoxicillin, and combined drug (212).

TEM-1 is a common beta-lactamase enzyme among gram-negative bacteria, particularly *Enterobacteriaceae* (1). It is the most common plasmid-mediated beta-lactamase which is a class A serine hydrolase (227-230). TEM-1 is also the most prevalence of TEM type in beta-lactamase producing isolates in *H. influenzae*. For epidemiology study, bla_{TEM} gene is known to be transposable, which $bla_{\text{TEM-1}}$ associated with transposon 2 and 3, therefore it can spread worldwide (231). In this study, the result from randomly selected in one of bla_{TEM} positive sequencing was TEM-1 type.

For BLNAR isolates, it is difficult to detect them in routine clinical microbiology laboratory because they must be beta-lactamase negative and has ampicillin-MIC above CLSI resistant breakpoint of >4 µg/ml (1). In contrast, isolates with ampicillin-MIC 2 µg/ml are often included in BLNAR type (19). Ampicillin resistance in BLNAR isolates occur from decreasing affinity of beta-lactams for some eight types of PBP resulting in peptidoglycan synthesis inhibition (39, 203). *H. influenzae* has eight PBP i.e., 1A, 1B, 2, 3A, 3B, 4, 5 and 6. In 1987, the decreased affinity of [3H]PEN for PBP2, PBP3A, PBP3B, and/or PBP4 in BLNAR isolates was reported (38). In 1990, the relationship between altered PBP3A, PBP3B and ampicillin resistance was confirmed by transformation experiment with chromosomal DNA from BLNAR isolates (178). In 1992, the determinants of beta-lactam resistance which mutations in the transpeptidase domain of *ftsI* encoded PBP3 were correlated with

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resistance (177). Therefore, PBP3 amino acid substitutions correlated with beta-lactam resistance by both genetic analysis and transformation studies (22-24, 212). In this study, *ftsI* gene amplification in BLNAR isolates was performed and selected 10 isolates according to various MIC values. The nucleotide sequences of *ftsI* gene in 10 isolates between 796 and 1,741 bp which encoded transpeptidase region of PBP3 were determined (21). The deduced amino acid sequences for BLNAR isolates were compared with *H. Influenzae* Rd isolates between amino acids 295-580. Therefore, BLNAR isolates have amino acid substitution mutations surrounding the conserved STVK (Ser327-Thr-Val-Lys, SSN (Ser379-Ser-Asn) and KTG (Lys512-Thr-Gly) motifs which were reported similar to other studies (16, 22, 25, 26).

There were various substitutions in *ftsI* gene, which Ubakata et al. (21) classified into three groups i.e., group I: R517H, group II: N526K and group III: three amino acids M377I, S385T, and L389F, respectively, in addition to N526K. In 2002, 108 *H. influenzae* isolates that were ampicillin resistant and had reduced susceptibilities to cephalosporins were investigated (22). The majority of isolates were within group II which they were further subdivided into 4 categories, group IIa: isolates have only N526K change; group IIb: isolates also have A502V in addition to N526K, with or without these other changes: G490Q, A437S, M377L and D350N; group IIc: isolates have N526K change coupled with A502T and group IId: isolates contained a single type of I449V in addition to D526N change (22). In 2003, (24) amino acid substitutions in *ftsI* gene similarly to several substitutions in previous reports (21, 22) were found. However, new substitutions e.g., A337V, T443A, R501L, V511A, Y528H and T532S were also found. These data indicated that amino acid substitutions in BLNAR isolates occur in many situations in several countries (223). Therefore, BLNAR isolates were highly variable in PBP3 mutations and certain mutation profiles were country specific (232). In this study, BLNAR isolates had various patterns of multiple PBP3 amino acid substitutions. We classified them into three groups (I, II, and III) according to criteria proposed by Ubukata et al (21). All of our BLNAR isolates had N526K substitution of Ubukata group II (21). Moreover, none of these BLNAR isolates had R517H of Ubukata group I and additional M377I, S385T and L389F substitutions of Ubukata group III. These results support other

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studies where isolates with R517H are generally less common and group III isolates are rare outside Japan (1, 25, 232, 233). Our results correlated with study by Garcia-Cobos et al. which R517H and N526K were also prevalent in Spanish isolates, with N526K substitution was the most frequent (223). The result from site-directed mutagenesis found that amino acid substitutions in R517H, N526K, S385T and L389F played important roles in beta-lactam resistance (212). In addition, Ubakata, et al. suggested that N526K may lead to neutral amino acid (Asn) change to basic amino acid (Lys) which affected transpeptidase domain structure (21).

Other mutations in PBP3 of BLNAR isolates also caused beta-lactam resistance (212). BLNAR-associated substitutions particularly common in our isolates were D350N (n=5), G490E (n=5), A502V/T (n=5) and N526K (n=10). Some isolates have A368T (n=1), A530S (n=3), V547I (n=3), V562L (n=2), N569S (n=3) substitutions which are infrequently found (1, 232, 233). To our knowledge M314V (n=1) and A561E (n=1) have not been previously reported. These amino acid substitutions led to distinct 7 patterns of substitutions which only one pattern was similar to *H. influenzae* PittEE (234). Amino acid substitutions in G490E, A502V, N526K, V547I, N569S were found in 2 isolates with the same ampicillin-MIC (2 µg/ml) while amino acid substitutions in M314V, D350N, A502V, N526K, V547I, N569S were found in 1 isolate (MIC= 2 µg/ml). Between these 2 different patterns, they were not different in ampicillin-MIC which correlated well with a study in 2011, which no correlation between ampicillin-MIC and the number of additional substitutions in group II isolates was observed (220). Almost BLNAR studies suggested that multiple changes in PBP3 lead to PBP3 low affinity binding to beta-lactams due to complication in secondary structural relationships (212). Between these two amino acid substitution patterns, they were different in two amino acid substitutions at position 314 from methionine changed to valine and position 350 from aspartic acid changed to asparagine. From previous study, changes in D350N and S357N were often found in almost all group II BLNAR isolates (21), while change in M314V has not been reported. Although the isolates containing M314V had MIC 2 µg/ml but this mutation may involve in beta-lactam resistance in BLNAR isolates. Because M314V was detected near Ser-Thr-Val-Lys (STVK) motif, it may locate at

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the active-site pocket surrounded STVK, SSN and KTG motifs similar to other substitutions in Ile-377, Thr-385, Phe-389, His-517 and Lys-526 (21). M314V substitution alters the residue size from 131.19 in methionine to 99.14 in valine. Therefore, its molecular weight was decreased similar to previous substitutions in M377I and M391I, which amino acid change from methionine to isoleucine might lead to low affinity between beta-lactams and PBP due to reduced amino acid residue size (218).

Structure of amino acid may result in resistant property e.g., mutation in PBP2x of *S. pneumoniae* revealed that mutation in position 338 from threonine to glycine, alanine, proline or valine showed the absence of hydroxyl group therefore, it drastically lowered the acylation efficiency of PBP2x for beta-lactams (235), although this residue did not directly contact with antibiotic (13). In this study, mutation in methionine-314 to valine showed the absence of sulphur group at this position which may affect structure of PBP3. As methionine and valine are non-polar amino acid therefore, this mutation did not alter the residue charge. Moreover, the effect of amino acid substitutions on PBP3 in *H. influenzae* can be examined by using molecular model in transpeptidase region encoded with *ftsI* in *H. influenzae*. This model was constructed based on structure of *S. pneumoniae* PBP2x (236). Two amino acid sequences of PBP3 in *H. influenzae* and PBP2x in *S. pneumoniae* had 29% homology which was enough to allow the region of *ftsI* gene product to be modelled. When the part of *H. influenzae* PBP3 was matched to PBP2X, the first two motifs had similar structure. But amino acid beyond Ser-446 was not be modelled because the level of amino acid in PBP3 was non-identity with PBP2x. Previous study, investigation of mutation at S357N compared with PBP2x via the SWISS-MODEL (24). Change from serine to asparagine provided steric hindrance with Val-362 side chain and affected the position of loop V362–P366 adjacent to active site causing direct interference with substrate binding by this residue (24). Therefore, further study in M314V by this model may the way to support beta-lactam resistance in BLNAR isolates. Amino acid substitutions in N526K and V562L were found in two isolates which showed the same ampicillin-MIC (4 µg/ml). Other substitutions were found in one isolate with D350N, A502T and N526K; and another isolate with A368T, A502T and N526K. These latter

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two amino acid substitutions also showed ampicillin-MIC (2 µg/ml). This result may indicate that amino acid substitution in V562L may play an important role in increased resistance to beta-lactams. In this study, ampicillin-MIC of isolate containing V562L correlated with the other study which ampicillin-MIC ranged 2-8 µg/ml was found in their isolates with V562L. Interestingly, this mutation coupled with other mutations showed cefotiam-MIC range 4-≥16 µg/ml (237). Then amino acid substitutions in D350N, G490E, N526K, A530S and A561E showed ampicillin-MIC of 16 µg/ml while substitution in D350N, G490E, N526K and A530S showed ampicillin-MIC of 8 µg/ml. There was one substitution difference at A561E which located near position 562, therefore substitution in position 561 from alanine to serine may lead to beta-lactam resistance. A561E substitution altered both the residue size and charges which the residue size from 71.09 in alanine changed to 129.12 in glutamic acid. It may be possible that glutamic acid has a higher molecular weight than alanine which may affect PBP3 structure causing unwanted steric hindrance. A561E also altered surface charge from neutral to acidic amino acid which may affect the structure of PBP3 binding to beta-lactams.

Amino acid substitution patterns may lead to occurrence of new group or subgroups which is necessary to investigate BLNAR diversity. The study in these patterns yearly is useful for data about diversity in Thailand such as France study (22). Study in France yearly for 14 years found group I, subgroups IIa and IIb existed as early as 1988. Subgroups IIc and IId had been in circulation since 1989 and 1991, respectively. As six new patterns appeared since 1999 which one of these new patterns became predominant in subgroup IIb while the others remained a minority. Many diversities in their study were observed in 1999 with 9 profiles from 26 isolates and 11 profiles from 39 isolates in 2000 (22).

To clarify relationship between mutations of PBP3 in BLNAR isolates and beta-lactam resistance, site-directed mutagenesis in single and multiple amino acid mutations at position 314 and 561 into PBP3 of beta-lactam susceptible RD isolates and MIC determination may need further investigation.

The result from restriction enzyme analysis i.e., *Sau*3AI and *Afa*I suggested that *Sau*3AI could not differentiate mutations occur in BLNAR isolates

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while *Afa*I may be used for roughly grouping in epidemiological study. This result may correlate with study in 2010, which the diversity in BLNAR and BLPACR isolates selected from each amino acid substitution subgroup were examined by PFGE digested with *Sma*I (2). They found PFGE profiles were greatly diverse and could not be classified into groups. In addition, the isolates that shared an amino acid substitution subgroup and had the same resistance type gave difference in PFGE profiles. This diversity showed the resistant isolates were clonally different from each other. Therefore, increasing BLNAR isolates tested and additional restriction enzyme investigation and molecular typing technique e.g., PFGE analysis in BLNAR isolates is necessary to further investigation for recognition the clonally spread. Data from geographical location, age of patients and date of specimen data will help support clonal spread. Moreover, BLNAR isolates may also transfer mutated *ftsI* gene to susceptible (BLNAS) isolates via horizontal gene transfer, this situation may occur via the USSs (uptake signal sequences) in donor DNA (238). Because of *H. influenzae* is naturally competent for DNA uptake which the uptake of DNA is mediated by the presence of USSs. The sequence of USS in *H. influenzae* is 5'-AAGTGCGGT-3' (239). It exists in middle and just downstream of *ftsI* gene (238). Previous study showed that USSs also exist in genomes of other species in family *Pasteurellaceae*, therefore this element is used for DNA uptake and DNA exchange between *H. influenzae* and related species (238). This situation can cause BLNAR acquiring resistant gene and leading to a rapidly increases in prevalence of this isolates. Therefore, BLNAR isolates from other countries may transfer mutated *ftsI* gene to *H. influenzae* in our country which, lead to a need in monitoring the prevalence of BLNAR isolates in worldwide. In this study, amoxicillin/clavulanic acid, cefotaxime, ceftriaxone and ceftazidime still show their good activity against both beta-lactamase producing and BLNAR isolates of *H. influenzae*. But the frequent use or misuse of amoxicillin-clavulanate and oral cephalosporins to treat respiratory tract infection may influence the development of BLNAR and BLPACR *H. influenzae* phenotype. Therefore, it is important to monitor the increased occurrence and spread of BLNAR and BLPACR isolates for appropriate antibiotic therapies. Moreover susceptibility testing with other cephalosporins is also necessary to further investigate the other drug

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resistance isolates e.g., amino acid substitution in S357N resulted in cefuroxime resistant isolates (24).

Overall study, beta-lactamase producing *H. influenzae* isolates remain high in Thailand while BLNAR isolates increased from 2008 to 2010. Therefore, the remaining of beta-lactamase producing and the emergence of BLNAR isolates are an important problem in antibiotic resistance. These isolates can also spread via clonal spread and horizontal gene transfer. Therefore, appropriate antibiotic use is important. This reason leads development of natural products from kaffir lime leaf and kaffir lime oils. Their antioxidant (240), repellent (241) and antiviral (242) as well as antibacterial activities were found. The most antibacterial effect of kaffir lime leaf and kaffir lime oils was previously reported for food-borne microorganisms e.g., *Bacillus cereus*, *Staphylococcus aureus*, and *Salmonella* Typhi (243). In 2008, the antimicrobial activities of kaffor lime leaf and kaffir lime oils against *S. aureus, B. cereus*, *Listeria monocytogenes*, were also reported (244). These oils of kaffir lime also showed greater activity against 20 serotypes of *Salmonella* and 5 species in other enterobacteria, compared to other spice oils (29). Kaffir lime leaf oil showed high antibacterial activity against *S. aureus*, while moderate activity against *S.* Typhi (27). Kaffir lime leaf, and other oil tested also has strongest antibacterial activity against 5 isolates of *Propionibacterium acnes* (30). Moreover, kaffir lime oil also showed antibacterial activity against extended-spectrum beta-lactamase producing *Klebsiella pneumoniae* (31).

Our result showed great activity of kaffir lime leaf and kaffir lime oils to various kind of tested bacteria. Although, some result showed variation in each tested bacteria. The reason may be associated with method of experiment, type of pathogens and components in oil.

Some bacteria e.g., Group A, C, F streptococci, *S. pneumoniae* and *H. influenzae* showed large inhibition zone in kaffir lime leaf oil, they provided low MIC and MBC values for both kaffir lime leaf oil and kaffir lime oil. This result may involve in the method of experiment. Disk diffusion is a screening antibacterial activity method. This method is usually used as a preliminary check for antibacterial activity prior to more detailed study and has many factors involvement e.g., volume of

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oil placed on paper disks, thickness of agar, diffusion ability of oil in agar and variation in oil concentration (245). Therefore, this method has many factors involvement and not suitable for comparison the efficiency in each type of oils whereas broth microdilution method is used to measure the strength of antibacterial activity (245). The result in this study was related with a study in *P. acnes* which kaffir lime leaf had the strongest antibacterial activity than lemongrass oil, clove oil and michellia oil by agar diffusion method, while lemongrass and citronella oil showed the highest antibacterial effect by MIC examination (30). Therefore, examination in antibacterial activity of essential oils by disk diffusion method is not suitable.

Moreover, components in kaffir lime leaf showed an ability to diffuse from paper disk and solubilize in agar (203, 210). While components in kaffir lime oil may more soluble in broth than kaffir lime leaf oil and may have synergistic effect between these components resulting in the high efficiency in broth microdilution method. This study, kaffir lime oil's result in *S. aureus* (MRSA, MSSA) and *A. baumannii* correlated with a study in *B. cereus* which suggested that many components in kaffir lime oil than kaffir lime leaf oil may lead to synergistic effect between these components, therefore kaffir lime oil showed high antibacterial activity to *B. cereus* than kaffir lime leaf oil (246). In this study, citronellal (80.04%) is a major component in kaffir lime leaf oil which is different with the oil from New Caledonian in France. In New Caledonian, kaffir lime leaf oil contained terpinene-4-ol (13%), alpha-terpineol (7.6%), 1, 8-cineole (6.4%), and citronellol (6%) (247). While kaffir lime oil in this study contained limonene (40.65%), terpinene-4-ol (13.71%) and alpha-terpineol (13.2%). These components correlated with other study in Thailand which its components were limonene (30.7%), pinene (18.8%), terpinene-4-ol (10.6 %) and terpineol (8.4%) (247). Antibacterial activity of citronellal was tested to prove whether it was an active component in kaffir lime leaf oil. This result correlated with previous study, which citronellal was proven to be a major antibacterial compound in kaffir lime leaf against *B. cereus* by cytoplasmic membrane attraction (248). Cironellal is a second aliphatic terpene aldehyde with ten carbon atoms which its functional group is aldehyde. Aldehydes have powerful antimicrobial activity (28). When, aldehyde group conjugated to a carbon to carbon double bond, it will have highly electronegative

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arrangement (185). Increasing in electronegativity will lead to increased antibacterial activity because they interfered in biological processes of bacteria e.g., electron transfer and reacted with vital nitrogen components such as proteins and nucleic acids (185). Therefore, aldehydes can cause microorganism growth inhibition. This result suggested that essential oils composed of large number of active components, which the main component showed high activity e.g., citral, a main component in lemongrass oil, showed high activity than other minor components against several microorganisms tested e.g., *S. aureus*, *E. coli*, *B. subtilis* and *C. albicans* (201, 249-251).

The antibacterial activity of limonene and alpha-terpineol were tested to prove whether they were active components in kaffir lime oil. The result found that limonene did not show antibacterial activity, but alpha-terpineol showed similar level antibacterial activity to kaffir lime oil. Our result was in contrast with other previous fungus study, which pure limonene at 25 µl/l had effective inhibition of *Fusarium verticillioides* (252). While minor components, alpha-terpineol is a monoterpene alcohol. The antibacterial activity of alcohol may occur by either protein denaturing agents (253) or dehydrating agent. In addition, terpinene-4-ol, another minor component in kaffir lime oil may have antibacterial activity because it shows very efficient antibacterial property in previous study (254). This result correlated with previous report, which antimicrobial activity against gram-positive bacteria and *Saccharomyces cerevisiae* of certain essential oils from aromatic plants occur by minor constituents (255).

The difference in each pathogen, both Gram positive and negative bacteria were performed in this study. Although, Gram positive bacteria is more often sensitive to essential oils than Gram-negative bacteria due to outer membrane barriers (245) and cell wall structure of Gram-negative bacteria is constituted with Lipopolysaccharides (LPS). Therefore, this component avoids the accumulation of oils on cell membrane (245). But in this study *H. influenzae* was the most sensitive to these oils therefore, gram-reaction may not involve in susceptibility of kaffir lime leaf and kaffir lime oils. Moreover, kaffir lime oil showed good effective antibacterial activity to important pathogens with drug resistant in this study e.g., *S. aureus* (MSSA and MRSA) and *A.*

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baumannii. Although MIC and MBC results varied between tested bacteria, in most cases, MIC was equivalent to MBC, indicating a bactericidal action of oils.

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CHAPTER VI
CONCLUSIONS

The present study can be concluded as follows:

1. For antimicrobial susceptibility of 100 beta-lactamase producing *H. influenzae* isolates tested to 9 antimicrobial agents, they were susceptible to cefotaxime, ceftriaxone, ciprofloxacin (100% each), followed by cefuroxime (96%), amoxicillin/clavulanic acid (88%), ceftazidime (67%), tetracycline (47%), and trimethoprim-sulphamethoxazole (28%). None of the isolates tested was susceptible to ampicillin. Twenty-six BLNAR *H. influenzae* isolates were susceptible to ampicillin (0%), amoxicillin/clavulanic acid (87.5%), cefotaxime (87.5%), ceftriaxone (87.5%), ceftazidime (87.5%), cefuroxime (41.67%), ciprofloxacin (100%), tetracycline (62.5%) and trimethoprim-sulphamethoxazole (29.17%).

2. Ampicillin-MIC values of 100 beta-lactamase producing *H. influenzae* isolates ranged from 8->512 µg/ml, whereas ampicillin-MIC values ranged from 2-16 µg/ml and amoxicillin/clavulanic acid-MIC values ranged from 0.75-4 µg/ml in BLNAR isolates.

3. By PCR technique, 99% of bla_{TEM} and 1% of $bla_{\text{ROB-1}}$ genes were found in beta-lactamase producing isolates. No isolate was positive for both bla_{TEM} and $bla_{\text{ROB-1}}$ genes. Prevalence of BLNAR isolates was found i.e., 7 isolates (3.95%) in 2008, 6 isolates (3.32%) in 2009 and 13 isolates (8.39%) in 2010.

4. For specifying TEM type of beta-lactamase gene by DNA sequence analysis in one randomly selected isolate of bla_{TEM} positive, 100% nucleotide and amino acid sequences identity with the published sequence of $bla_{\text{TEM-1}}$ gene was found.

5. For detection of mutations in *ftsI* gene encoded PBP3 in 10 BLNAR isolates. Amino acid substitutions were M314V, A368T, D350N, G490E, A502V/T, N526K, A530S, V547I, A561E, V562L and N569S. There were 7 patterns of amino acid substitutions, which all isolates were group II according to Ubukata et al (21).

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There was 1 pattern, which was correlated with *ftsI* gene in *H. influenzae* PittEE with 100% homology, while the other 6 patterns were discovered in this study. In addition, mutations at positions M314V and A561E were identified in this study for the first time.

6. For restriction enzyme analysis in BLNAR isolates by using *Sau*3AI and *Afa*I enzymes. *Sau*3AI could not differentiate these mutations, while *Afa*I may roughly differentiate group of these isolates.

7. For antibacterial activity of kaffir lime leaf and kaffir lime oils to various kind of respiratory tract pathogens, both oils showed antibacterial activity by disk diffusion in *H. influenzae*, followed by *S. pneumoniae*, Group A, F, G, C, B streptococci, *S. aureus* (MRSA, MSSA) and *A. baumannii*, respectively for kaffir lime leaf oil. For kaffir lime oil large inhibition zones in decreasing order were found in *S. aureus* (MRSA, MSSA), *A. baumannii*, *S. pneumoniae*, Group F streptococci, *H. influenzae*, Group A, G, C and B streptococci, respectively. The lowest MIC and MBC values of kaffir lime leaf and kaffir lime oils against *H. influenzae* were decreased among tested bacteria.

Citronellal, a major constituent in kaffir lime leaf oil, showed good antibacterial activity similar to kaffir lime leaf oil in all tested bacteria. Limonene, a constituent in kaffir lime oil showed low level of antibacterial activity therefore, citronellal may be the active ingredient in kaffir lime leaf oil whereas limonene may need other constituents in this oil for synergistic effect.

8. Antibacterial activity of kaffir lime leaf and kaffir lime oils was stable upon storage at room temperature for 4 months.

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APPENDICES

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APPENDIX A

Culture media

1. Chocolate agar

Prepare brain heart infusion agar according to instruction given on the label of dehydrated powder by suspending 52 g of the powder in 1 L of purified water. Then dextrose 1.25 g and 3 g of agar are added to perform in 1 L of total volume. It is convenient to prepare 500 ml of molten agar in a 1 liter flask. Autoclave at 121 °C for 15 min, then at a temperature (75-80 °C), defibrinated sheep blood is added (5%; 25 ml sheep blood is added to 500 ml of agar). After the temperature cool down to 50 °C, 30 ml of sterile coconut juice is added to this media to provide necessary supplement for growth. Then dispense into 15 x 100 mm Petri dishes. Allow to solidify, place in plastic bag and store at 4 °C.

2. Sheep blood agar

Prepare brain heart infusion according to instruction given on the label of dehydrated powder by suspending 52 g of the powder in 1 L of purified water. It is convenient to prepare 500 ml of molten agar in a 1 liter flask. Autoclave at 121 °C for 15 min, cool to 50 °C and defibrinated sheep blood is added (5%; 25 ml sheep blood is add to 500 ml of agar). Dispense into 15 x 100 mm Petri dishes. Allow to solidify, place in plastic bag and store at 4 °C. The plates should appear a bright red color. If the agars appear dark red, because the blood is added when the agar is still very hot, discard the medium and prepare another batch.

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3. Mueller-Hinton agar

Prepare Mueller-Hinton agar according to instruction on the label of dehydrated medium by suspending 38 g of the powder in 1 L of purified water. Autoclave at 121 °C for 15 min. Dispense into 15 x 100 mm Petri dishes. Allow to solidify, place in plastic bags and store at 4 °C.

4. *Haemophilus* Test Medium agar

Prepare a fresh hematin stock solution by dissolving 50 mg of hematin powder in 100 ml of 0.01 mol/L NaOH with heat and stirring until the powder is thoroughly dissolved. Add 30 ml of the hematin stock solution and 5 g of yeast extract to 1 L of Mueller-Hinton agar and autoclave at 121 °C for 15 min. After autoclaving and cooling, add 3 ml of nicotinamide adenine dinucleotide (NAD) stock solution (50 mg of NAD dissolved in 10 ml of distilled water (filter sterilized) aseptically. Dispense into 15 x 100 mm Petri dishes. Allow to solidify, place in plastic bag and store at 4 °C.

5. *Haemophilus* Test Medium broth

Prepare cation-adjusted Mueller-Hinton broth by suspending 22 g of the powder in 1 L of purified water. Bring to the boil to dissolve. Sterilize by autoclaving at 121° C for 15 min. Cool to 50°C and aseptically add the contents of *Haemophilus* Test Medium supplement SR0158 (Oxoid Ltd, USA), as directed. *Haemophilus* Test Medium supplement SR0158 is prepared by dissolving 15 mg of 1 vial of this supplement in 2 ml sterile distilled water then mix well. 1 vial of *Haemophilus* Test Medium supplement can be added to 500 ml of cation-adjusted Mueller-Hinton broth.

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6. *Haemophilus* Test Medium (HTM) broth with Tween 20

Prepare HTM broth according to above method. Tween 20 is added to the first step of cation-adjusted Mueller-Hinton broth preparation by adding 0.25% of Tween 20 and boil for 1 min to completely dissolve before autoclaving.

7. Cation-adjusted Mueller-Hinton broth (CAMHB) with Tween 20

Suspend 22 g of the powder in 1 L of purified water then add 0.25% of Tween 20 in the combination. Mix thoroughly and heat with frequent agitation and boil for 1 min to completely dissolve the powder and then autoclave at 121 °C for 15 min.

8. Cation-adjusted Mueller-Hinton broth (CAMHB) with lysed horse blood (LHB) and Tween 20

Cation-adjusted Mueller-Hinton broth (CAMHB) with Tween 20 is prepared according to previously described method. Before use, 2-5% of LHB is added to this medium to obtain cation-adjusted Mueller-Hinton Broth (CAMHB) with lysed horse blood (LHB) and Tween 20.

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APPENDIX B

Complementary position between primers and their DNA sequences

1. Nucleotide sequence of *bla*$_{TEM}$ gene (23641-24960 nucleotides of *E. coli* plasmid pC15-1a; *bla*$_{TEM-1}$ gene, GenBank Accession No. NC005327). Nucleotide sequences complementary with TEM primers are boldface. The start codon of *bla*$_{TEM-1}$ is indicated by horizontal arrow. The stop codon of *bla*$_{TEM-1}$ and tnpR are indicated by asterisks.

//

tnpR

23641 tgtcggctgt ggcacaggct gaacgccgga ggatcctaga acgcacgaat gagggccgac

23701 aggaagcaaa gctgaaagga atcaaatttg gccgcaggcg taccgtggac aggaacgtcg

23761 tgctgacgct tcatcagaag ggcactggtg caacggaaat tgctcatcag ctcagtattg

23821 cccgctccac ggtttataaa attcttgaag acgaaagggc ctcgtga*tac gcctattttt

23881 ataggttaat gtcatgataa taatggtttc ttagacgtca ggtggcactt ttcggggaaa

23941 tgtgcgcgga acccctattt gtttattttt ctaaatacat tcaaatatgt atccgctcat

bla$_{TEM-1}$

24001 gagacaataa ccctggtaaa tgcttcaata atattgaaaa aggaagagt**a** **→tgagtattca**

TEM F

24061 **acattttcg**t gtcgcccttа ttccctttтt tgcggcattt tgccttcctg tttttgctca

24121 cccagaaacg ctggtgaaag taaaagatgc tgaagatcag ttgggtgcac gagtgggtta

24181 catcgaactg gatctcaaca gcggtaagat ccttgagagt tttcgccccg aagaacgttt

24241 tccaatgatg agcactttta aagttctgct atgtggtgcg gtattatccc gtgttgacgc

24301 cgggcaagag caactcggtc gccgcataca ctattctcag aatgacttgg ttgagtactc

24361 accagtcaca gaaaagcatc ttacggatgg catgacagta agagaattat gcagtgctgc

24421 cataaccatg agtgataaca ctgctgccaa cttacttctg acaacgatcg gaggaccgaa

24481 ggagctaacc gctttttttgc acaacatggg ggatcatgta actcgccttg atcgttggga

24541 accggagctg aatgaagcca taccaaacga cgagcgtgac accacgatgc ctgcagcaat

24601 ggcaacaacg ttgcgcaaac tattaactgg cgaactactt actctagctt cccggcaaca

24661 attaatagac tggatggagg cggataaagt tgcaggacca cttctgcgct cggcccttcc

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24721 ggctggctgg tttattgctg ataaatctgg agccggtgag cgtgggtctc gcggtatcat
24781 tgcagcactg gggccagatg gtaagccctc ccgtatcgta gttatctaca cgacggggag
24841 tcaggcaact atggatgaac gaaatagaca gatcgctgag ataggtgcct cactgattaa
      TEM R *
24901 gcattggtaa ctgtcagacc aagtttactc atatatactt tagattgatt taaaacttca
//
```

2. Nucleotide sequence of *ftsI* gene (1197840-1199672 nucleotides of *Haemophilus influenzae* Rd KW20, complete genome, GenBank Accession No. NC_000907). Nucleotide sequences complementary with *ftsI* primers are boldface.

```
1197840 atggtgaaat ttaattcctc gcgtaaatca ggtaagtcaa aaaaaacaat tagaaaattg
1197900 accgcacctga aactgtaaa gcaaaacaag cctcaaaagg tgtttgaaaa atgctttatg
1197960 cgtggacgtt atatgctttc tacggttctt attttacttg gcctgtgtgc tttagtcgca
1198020 cgagcagctt atgttcaatc tattaatgcc gatacgttat cgaatgaagc ggataagcgt
1198080 tctttgcgta aagatgaagt attatcggtg cgtggttcta ttttagatcg taatggtcag
1198140 cttttatctg taagcgtgcc gatgagcgcg attgtggcag atccaaaaac gatgttgaag
1198200 gaaaattcgc ttgcggataa agaacgaatt gcagctttag ccgaagaatt aggtatgact
1198260 gaaaatgatt tagtgaaaaa aattgagaaa aattctaaat ctggttattt gtatttagca
1198320 cgtcaagttg aattaagtaa agctaactat attcgtagat taaaaattaa gggtattatt
1198380 ttagaaacag agcatcgccg tttttatcct cgtgtagaag aagctgcaca cgtggtgggt
1198440 tatacggata ttgatggaaa tggtattgaa ggcattgaga aaagttttaa ttccctgctt
1198500 gttggtaaag acggttcacg tactgttcgt aaagataaac gtgggaatat tgttgcacat
1198560 atctccgatg agaaaaaata tgatgcacaa gatgttacct taagtatcga tgaaaaattg
1198620 caatctatgg tgtatcgtga gattaaaaag gcggtgtctg agaataatgc tgagtctggt
                                    ftsI                ftsI F
1198680 actgcggtgt tagttgatgt tcgaacaggg→gaagtgttag ctatggcgac tgcgccctct
1198740 tataatccaa acaaccgtgt cggcgtgaaa tcagagttaa tgcgtaaccg tgcaattacc
1198800 gatacttttg agccaggttc tacggtaaaa cctttcgttg ttttaaccgc acttcaacga
1198860 ggtgtagtta aacgagatga aattattgat actacgtcct ttaaattaag cggtaaagaa
1198920 attgtggacg ttgcaccacg tgctcagcaa actttagacg agattttaat gaactctagt
1198980 aaccgtggtg taagtcgtct tgcattacgt atgccaccta gtgcattaat ggaaacttat
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1199040 caaaatgcag gtttaagtaa accgacagat ttaggcttga tcggagagca agttgggatt

1199100 ttgaatgcaa atcgtaaacg ctgggcagat attgagcgtg caacagtcgc ttatggttat

1199160 ggtattactg cgacaccttt acaaattgct cgtgcctatg caacccttgg tagtttcggt

1199220 gtttatcgtc cgctttctat cactaaagtt gatccgccag ttattgggaa acgggttttc

1199280 tctgaaaaaa taactaaaga tattgtggga attttagaga aagtagcaat taaaaataaa

1199340 cgcgcaatgg tggaaggcta ccgtgtcggc gtaaaaacag gtacggcacg taagattgaa

1199400 aatggacatt atgtaaataa gtatgtggca tttactgcgg gtattgcacc aattagtgat

1199460 cctcgttatg cattagtggt tttgatcaat gatccaaaag caggagaata ttatggtggt

ftsI R

1199520 gcggtttctg cccctgtatt ctctaacatt atgggctatg cgttacgtg**c aaatgctatt**

1199580 **ccgcaaga**tg ctgaagcagc tgaaaacaca acaacgaaaa gtgcaaaacg tattgtttat

1199640 attggcgaac acaagaatca aaaagtgaat taa

3. Nucleotide sequence of *ftsI* gene (1197840-1199672 nucleotides of *Haemophilus influenzae* Rd KW20, complete genome, GenBank Accession No. NC_000907). Nucleotide sequences complementary with *ftsI* walking primers are boldface.

1197840 atggtgaaat ttaattcctc gcgtaaatca ggtaagtcaa aaaaaacaat tagaaaattg

1197900 accgcacctga aactgtaaa gcaaaacaag cctcaaaagg tgtttgaaaa atgctttatg

1197960 cgtggacgtt atatgctttc tacggttctt attttacttg gcctgtgtgc tttagtcgca

1198020 cgagcagctt atgttcaatc tattaatgcc gatacgttat cgaatgaagc ggataagcgt

1198080 tctttgcgta aagatgaagt attatcggtg cgtggttcta ttttagatcg taatggtcag

1198140 cttttatctg taagcgtgcc gatgagcgcg attgtggcag atccaaaaac gatgttgaag

1198200 gaaaattcgc ttgcggataa agaacgaatt gcagctttag ccgaagaatt aggtatgact

1198260 gaaaatgatt tagtgaaaaa aattgagaaa aattctaaat ctggttattt gtatttagca

1198320 cgtcaagttg aattaagtaa agctaactat attcgtagat taaaaattaa gggtattatt

1198380 ttagaaacag agcatcgccg tttttatcct cgtgtagaag aagctgcaca cgtggtgggt

1198440 tatacggata ttgatggaaa tggtattgaa ggcattgaga aaagttttaa ttccctgctt

1198500 gttggtaaag acggttcacg tactgttcgt aaagataaac gtgggaatat tgttgcacat

1198560 atctccgatg agaaaaaata tgatgcacaa gatgttacct taagtatcga tgaaaaattg

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1198620 caatctatgg tgtatcgtga gattaaaaag gcggtgtctg agaataatgc tgagtctggt

1198680 actgcggtgt tagttgatgt tcgaacaggg gaagtgttag ctatggcgac tgcgccctct

1198740 tataatccaa acaaccgtgt cggcgtgaaa tcagagttaa tgcgtaaccg tgcaattacc

1198800 gatacttttg agccaggttc tacggtaaaa cctttcgttg ttttaaccgc acttcaacga

1198860 ggtgtagtta aacgagatga aattattgat actacgtcct ttaaattaag cggtaaagaa

1198920 attgtggacg ttgcaccacg tgctcagcaa actttagacg agattttaat gaactctagt

1198980 aaccgtggtg taagtcgtct tgcattacgt atgccaccta gtgcattaat ggaaacttat

1199040 caaaatgcag gtttaagtaa accgacagat ttaggcttga tcggagagca agttgggatt

1199100 ttgaatgcaa → *ftsI* at**cgtaaacg ctgggcagat a**ttgagcgtg caacagtcgc ttatggttat

1199160 ggtattactg cgacaccttt acaaattgct cgtgcct**atg caacccttgg tagttt**cggt

1199220 gtttatcgtc cgctttctat cactaaagtt gatccgccag ttattgggaa acgggttttc

1199280 tctgaaaaaa taactaaaga tattgtggga attttagaga aagtagcaat taaaaataaa

1199340 cgcgcaatgg tggaaggcta ccgtgtcggc gtaaaaacag gtacggcacg taagattgaa

1199400 aatggacatt atgtaaataa gtatgtggca tttactgcgg gtattgcacc aattagtgat

1199460 cctcgttatg cattagtggt tttgatcaat gatccaaaag caggagaata ttatggtggt

1199520 gcggtttctg cccctgtatt ctctaacatt atgggctatg cgttacgtgc aaatgctatt

1199580 ccgcaagatg ctgaagcagc tgaaaacaca acaacgaaaa gtgcaaaacg tattgtttat

1199640 attggcgaac acaagaatca aaaagtgaat taa

ftsI walking primer F

ftsI walking primer R

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APPENDIX C

Reagents for gel electrophoresis

1. 0.5M EDTA

Dissolve 146.12 g of EDTA in 200 ml of distilled water. Adjust the pH to 8.0 and autoclave. Store at room temperature for no longer than 1 year.

2. 50X TAE buffer

Dissolve 108 g of Tris base, 55 g of boric acid and 40 ml of 0.5M EDTA. Mix thoroughly and adjust the pH to 8.0. Then adjust the volume to 1 L. Store at room temperature for no longer than 1 year.

3. Loading dye (10X)

Dissolve 8 g of sucrose (40% final) in 10 ml of 0.5M EDTA (250 mM final). Add distilled water to 20 ml, dissolve sucrose completely and then add 0.15 g of bromphenol blue (1.5% final), autoclave, aliquot 1 ml into eppendorfs and store at room temperature.

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