CHAPTER 5

Results

5.1 Antimicrobial susceptibilities

Total 182 ESBL-producing isolates were tested for MICs of cefotaxime and ceftazidime. It was found that 181 (99.4%) were resistant to cefotaxime (MIC >2 µg/ml) whereas 140 (76.9%) were non-susceptible to ceftazidime (MIC ≥ 8 µg/ml) (Figure 5.1). The MIC_{50}/MIC_{90} of cefotaxime and ceftazidime were 128/512 and 16/128 µg/ml, respectively. The result showed that 156 (85.7%) of 182 isolates were cefotaxime-resistant and MICs of this drug was higher than MIC of ceftazidime. There were 42 isolates susceptible to ceftazidime. ESBL-producing *E. coli* were recovered most frequently from urine (65.4%), followed by pus (19.8%), sputum (6%), body fluid (4.4%) and blood (4.4%) specimens.

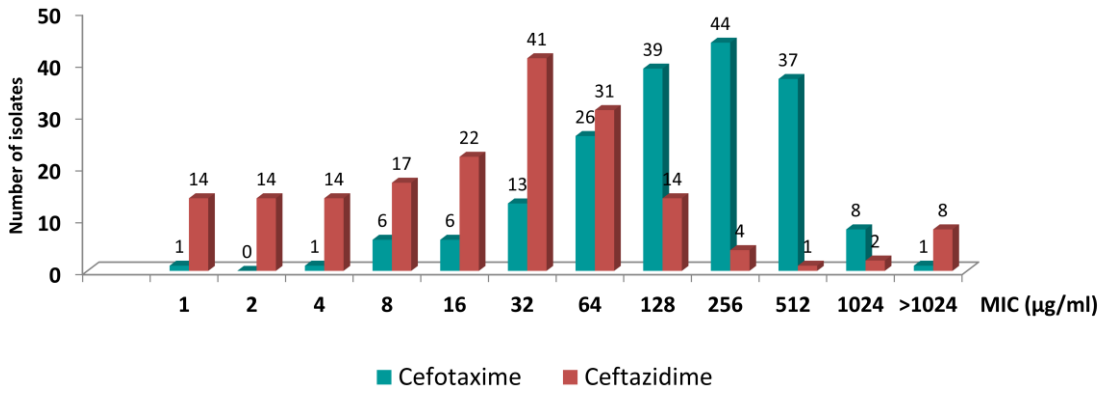


Figure 5.1 Minimal inhibitory concentration (MIC) distribution of cefotaxime and ceftazidime of β-lactamase producing *E. coli*. The breakpoints for being susceptible, intermediate, or resistant to cefotaxime are ≤1, 2 and ≥4 µg/ml respectively. The breakpoints for being susceptible, intermediate, or resistant to ceftazidime are ≤4, 8 and ≥16 µg/ml, respectively.

5.2 Molecular characterization

5.2.1 Multiplex PCR detection of ESBL and AmpC β-lactamase genes

1) Specificity test of multiplex primers

Total DNA samples of 7 *E. coli* strains carrying each identified ESBL and AmpC β-lactamase genes were used as positive controls for multiplex PCR throughout this study. Each strain carries different beta lactamase gene including *bla*$_{TEM3}$, *bla*$_{SHV5}$, *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-14}$, *bla*$_{CMY8b}$, *bla*$_{CMY2}$ and *bla*$_{DHA-1}$. All positive control strains were used for verifying the specificity of primers within three multiplex primer groups. All primers used in this study were previously designed and used for multiplex PCR assay detecting important beta-lactamase genes in Enterobacteriaceae (Dallenne *et al*., 2010). Multiplex I and Multiplex II were used for detecting ESBL genes while Multiplex III was used for detecting AmpC β-lactamase genes. Multiplex I primer group comprised 2 pairs of primers, Multi TS-T primers for detecting *bla*$_{TEM}$ variants and Multi TS-S primers for detecting *bla*$_{SHV}$ variants. Multiplex II primer group comprised 3 pairs of primers, Multi CTX-M-G1 for detecting *bla*$_{CTX-M-1\ subgroup}$ variants, Multi CTX-M-G2 for detecting *bla*$_{CTX-M-2\ subgroup}$ variants and Multi CTX-M-G9 for detecting *bla*$_{CTX-M9\ subgroup}$ variants. Multiplex III primer group comprised 3 pairs of primers, MultiCD-CMY-1 for detecting *bla*$_{CMY1}$ and other variants, MultiCD-CMY-2 for detecting *bla*$_{CMY2}$ and other variants, and MultiCD-DHA for detecting *bla*$_{DHA}$variants. Amplicons of the corresponding sizes were obtained from all control strains confirming the group specificity of the primers. Evaluation of specific group primer demonstrated high specificity for Multiplex I and III (Figure 5.2). Multiplex I PCR yielded two amplicons visualized on ararose gel with sizes of approximately 800 bp corresponding to *bla*$_{TEM}$ and 713 bp corresponding to *bla*$_{SHV}$, respectively. Multiplex III PCR yield three amplicons with sizes of 997 bp corresponding to *bla*$_{CMY2}$, 895 bp corresponding to *bla*$_{CMY1}$, and 538 bp corresponding to *bla*$_{DHA}$, respectively. Multiplex II PCR yielded 3 amplicons with sizes of approximately 688 bp corresponding to *bla*$_{CTX-M-1\ subgroup}$, 561 bp corresponding to *bla*$_{CTX-M-9\ subgroup}$, and 400-450 bp corresponding to *bla*$_{CTX-M-2\ subgroup}$. However, nucleotide sequencing of the 400-450 bp amplicon visualized on

agarose gel revealed the actual size of 360 bp. This sequence was not full PCR product. A small area at the upstream sequence could not be interpreted due to low yield PCR product and the initial reaction of nucleotide sequencing. Sequence analysis revealed high similarity (99% identical) to *bla*$_{\text{CTX-M-14}}$ gene and *bla*$_{\text{CTX-M-123}}$ that belong to the *bla*$_{\text{CTX-M-9 subgroup}}$ (Figure 5.3). Monoplex PCR using a single pair of primers specific to *bla*$_{\text{CTX-M-2 subgroup}}$ yielded this 400-450 bp amplicon whereas the primers specific to *bla*$_{\text{CTX-M-9 subgroup}}$ did not. This result suggests that the primers specific for *bla*$_{\text{CTX-M-2 subgroup}}$ can cross-react with other *bla*$_{\text{CTX-M-subgroup}}$ gene because nucleotide sequence of this primer was possible bind with CTX-M-9subgroup (CTX-M-9 and CTX-M-14) and CTX-M-123. Interestingly, the result in this study was found CTX-M-G1 primers were able to bind to the sequence of CTX-M-123 (Figure 5.3).

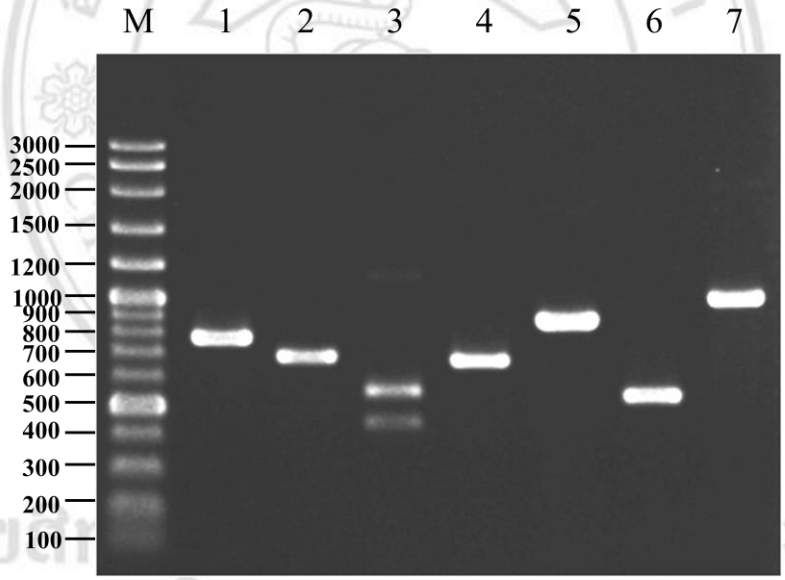
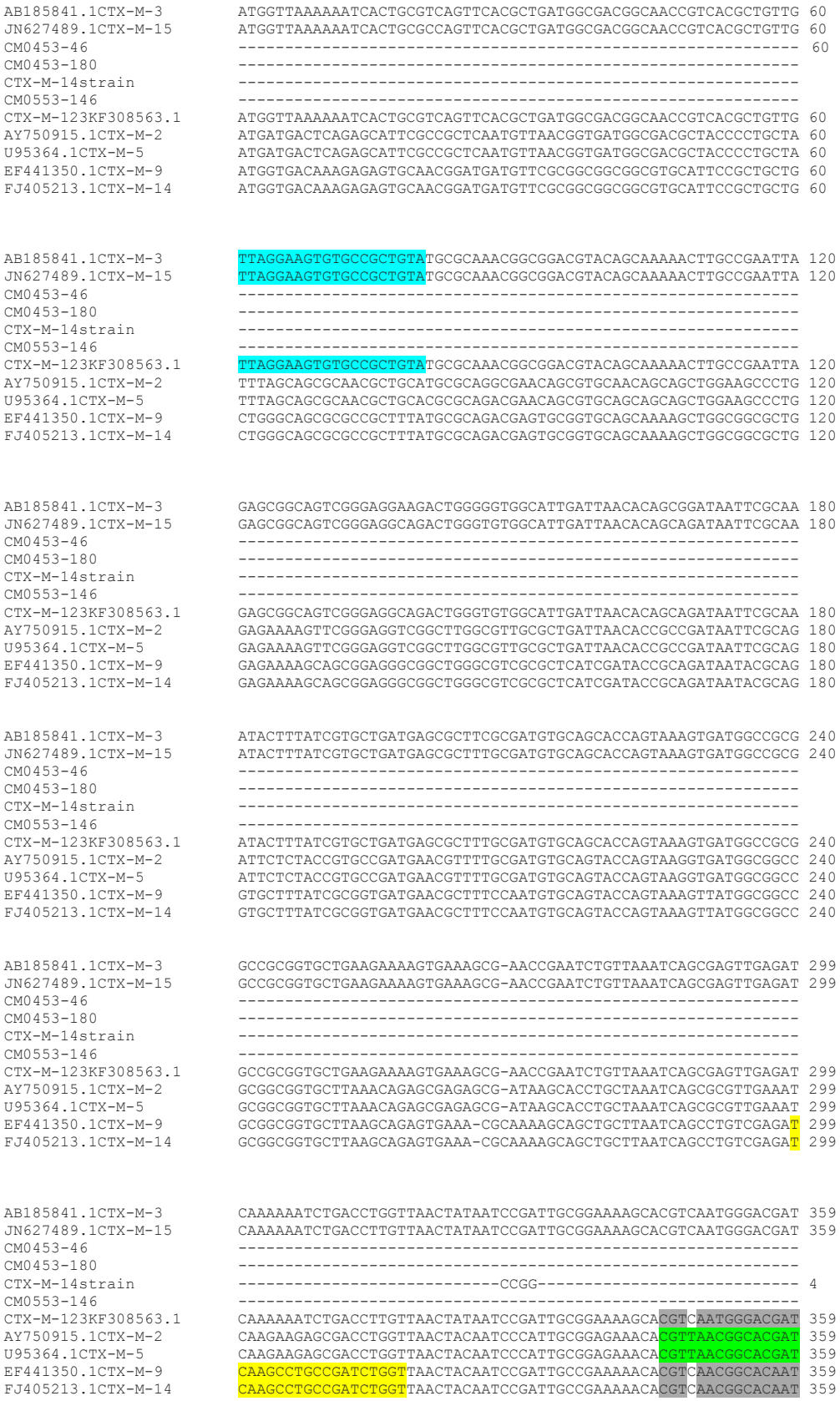


Figure 5.2 Amplification products of positive control strains. Multiplex PCR products were separated in 1% agarose gel. Lanes: 1, PCR product of *bla*$_{\text{TEM}}$ (800bp); 2, PCR product of *bla*$_{\text{SHV}}$ (713 bp); 3, PCR product of *bla*$_{\text{CTX-M-9subgroup}}$ (561 bp); 4, PCR product of *bla*$_{\text{CTX-M-1subgroup}}$ (688bp); 5, PCR product of *bla*$_{\text{CMY1}}$ (895bp); 6, PCR product of *bla*$_{\text{CMY2}}$ (538bp); 7, PCR product of *bla*$_{\text{DHA}}$ (997bp); M, 100bp DNA marker.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CM0453-180 ----------
CTX-M-14strain ----------
CM0553-146 ----------
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Figure 5.3 multiple sequence alignment: comparison of nucleotide sequence of CTX-M-123(unexpected PCR products) with CTX-M-123 from data base and other CTX-M. Unexpected PCR products that similar to CTX-M-123 and CTX-M-9subgroup are shown in red. Specific binding sites of MultiCTXM-G1, MultiCTXM-G2, MultiCTXM-G9 primer-pairs are highlighted in blue, green and yellow, respectively. Possible position that MultiCTXM-G2 primer-pair have cross-reaction with CTX-M-9subgroups and CTX-M-123 are highlight in gray.

2) Optimization of annealing temperature

Eight primer-pair for the genes of interest were combined into 3 multiplex PCR groups. This study was optimized in annealing temperature and final concentration of primer. Titration of annealing temperature at 56 to 65 ^{o}C was tested. The annealing temperature of 65^{o}C for Multiplex I (Figure 5.4) ,60^{o}C for Multiplex II (Figure 5.5) and 57^{o}C Multiplex III (Figure 5.6) were selected for subsequent studies.

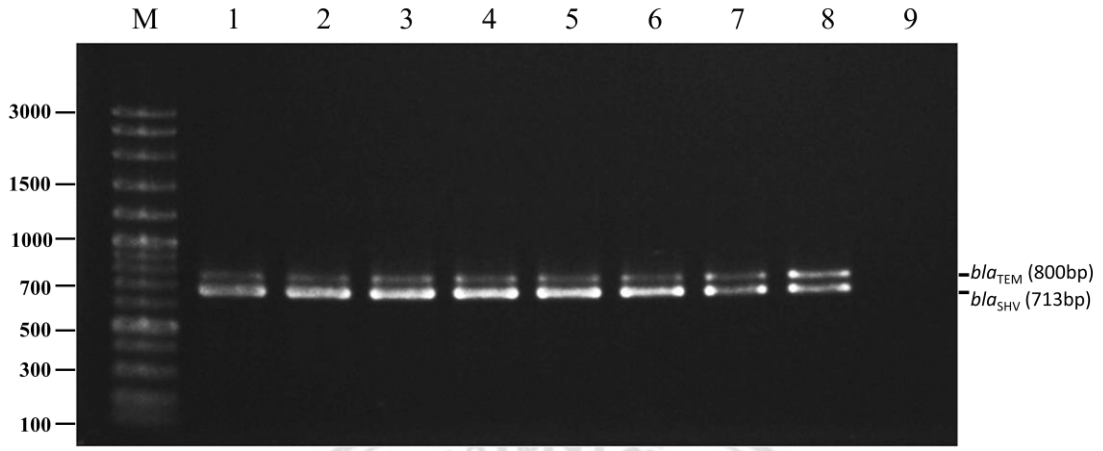

Figure 5.4 Multiplex I: Multiplex PCR product of *bla*$_{TEM}$ (800bp) and *bla*$_{SHV}$ (713bp) at various annealing temperatures. Lanes:1, 56^{o}C; 2, 56.9^{o}C; 3, 57.8^{o}C; 4, 59^{o}C; 5, 60^{o}C; 6, 61^{o}C; 7, 63.2^{o}C; 8, 65^{o}C; 9, negative control; M, 100bp DNA marker.

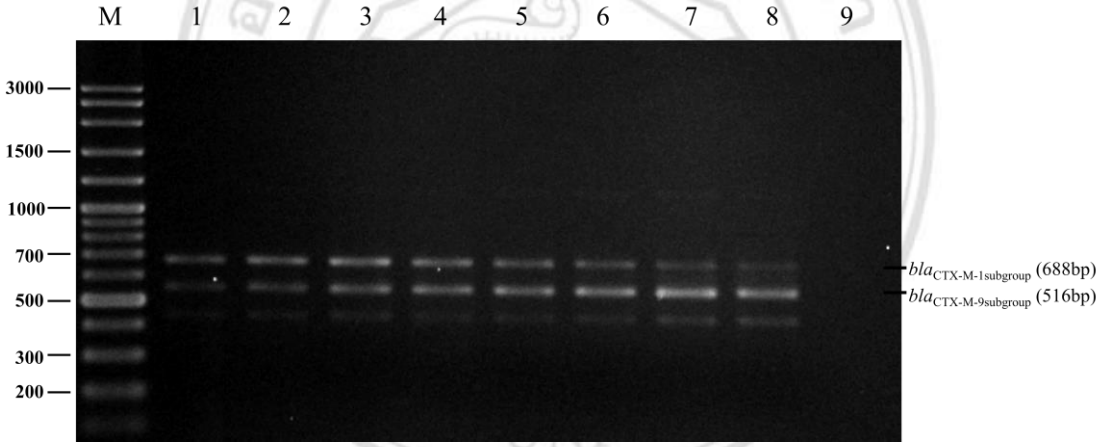

Figure 5.5 Multiplex II: Multiplex PCR product of *bla*$_{CTX\text{-}M\text{-}1subgroup}$ (688bp) and *bla*$_{CTX\text{-}M\text{-}9subgroup}$ (561 bp) at various annealing temperatures. Lanes:1, 56^{o}C; 2, 56.9^{o}C; 3, 57.8^{o}C; 4, 59^{o}C; 5, 60^{o}C; 6, 61^{o}C; 7, 63.2^{o}C; 8, 65^{o}C; 9, negative control; M, 100bp DNA marker .

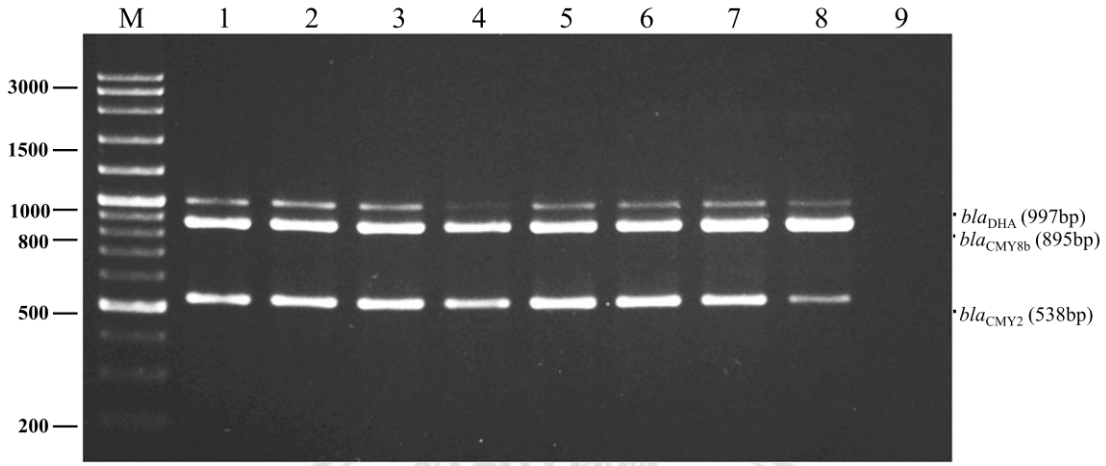

Figure 5.6 Multiplex III: Multiplex PCR product of *bla*$_{CMY8b}$ (895bp), *bla*$_{CMY2}$ (538bp) and *bla*$_{DHA}$ (997bp) at various annealing temperatures. Lanes:1, 56^{o}C; 2, 56.9^{o}C; 3, 57.8^{o}C; 4, 59^{o}C; 5, 60^{o}C; 6, 61^{o}C; 7, 63.2^{o}C; 8, 65^{o}C; 9, negative control; M, 100bp DNA marker.

3) Optimization of the primer concentration

To complete a higher yield of amplification products, various concentrations of these group-specific primers were tested. In this study was optimized in order to complete specific PCR products in the multiplex PCR, as follows: (i) Multiplex I, based on previously published report (Dallenne *et al*., 2010), the concentrations of Multi TS-T and Multi TS-S primer-pairs were tested at 0.2, 0.4 and 0.8 pmol/µl. The results showed those primers produced unbalanced yields of both PCR products at 0.2 pmol/µl and were not different amplified product at 0.4 and 0.8 pmol/µl. Therefore, the final optimal concentration of both primer pair for *bla*$_{TEM}$ and *bla*$_{SHV}$ were 0.4 pmol/µl (Figure 5.7). (ii) Multiplex II, the various concentration of were tested at 0.15, 0.3, 0.4 and 0.8 pmol/µl for Multi CTX-M-G1, at 0.1, 0.2, 0.3 and 0.8 pmol/µl for Multi CTX-M-G2 and at 0.2, 0.4, 0.4 and 0.8 pmol/µl for Multi CTX-M-G9. The result is demonstrated in Figure 5.8. The final concentration at 0.1 and 0.2 pmol/µl of Multi CTX-M-G2 primer was not detected unexpect band in 1% agrose gel electrophoresis. The higher concentrations (0.4 and 0.8 pmol/µl) of Multi CTX-M-G1 and Multi CTX-M-G9 were not different amplification products. Therefore, a final concentration at 0.4, 0.3 and 0.4 pmol/µl for Multi CTX-M-G1, Multi CTX-M-G2 and Multi CTX-M-G9 primer-pair, respectively were used in this study. (iii) Multiplex III, the

concentration of primers were tested at 0.1, 0.2 and 0.4 pmol/µl for MultiCD-CMY-1 and MultiCD-CMY-2 primer, at 0.25, 0.5 and 1 pmol/µl for MultiCD-DHA primer. The results of MultiCD-DHA primers showed lower PCR product at 0.5 and 1 pmol/µl (Figure 5.9). A final concentration at 0.25 pmol/µl was selected for further use in subsequent multiplex PCR. The optimal concentration of the three primer pair, for *bla*$_{CMY1}$, *bla*$_{CMY2}$and *bla*$_{DHA}$ were found to be 0.1, 0.1 and 0.25 pmol/µl, respectively.

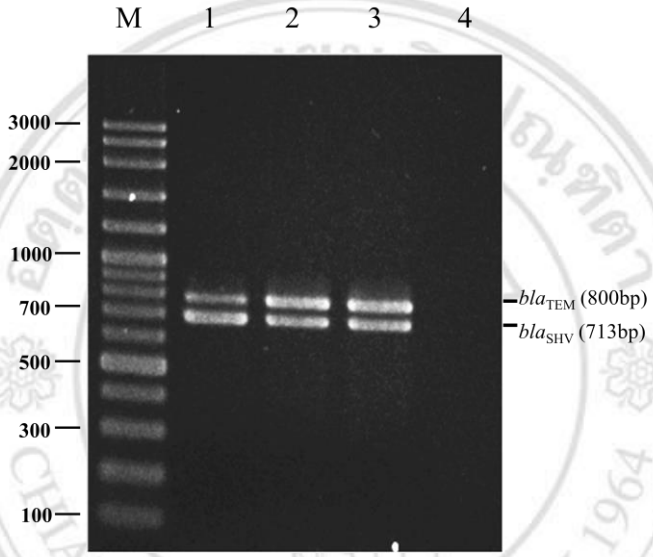

Figure 5.7 Multiplex I: Multiplex PCR products of *bla*$_{TEM}$ (800bp) and *bla*$_{SHV}$ (713bp) at different concentration of Multi TS-T and Multi TS-S primers-pair. Lanes: 1, 0.2 and 0.2 pmol/µl, respectively; 2, 0.4 and 0.4 pmol/µl, respectively; 3, 0.8 and 0.8 pmol/µl, respectively; 4, negative control; M, 100bp DNA marker

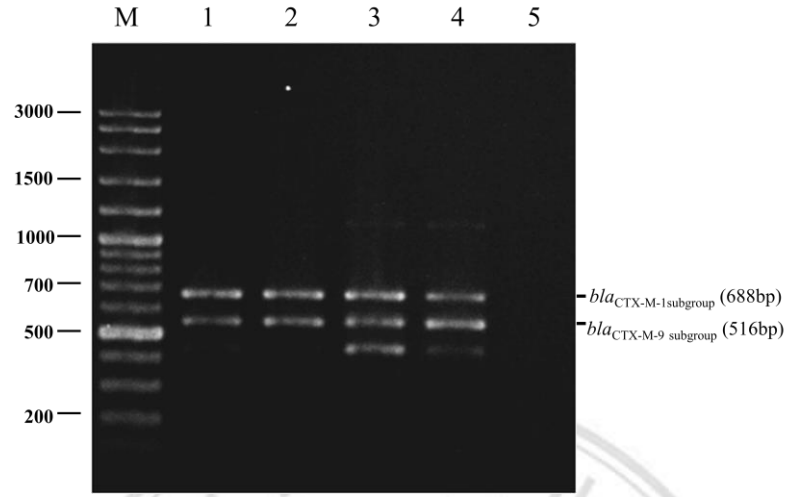

Figure 5.8 Multiplex II: Multiplex PCR products of *bla*$_{\text{CTX-M-1subgroup}}$ (688bp) and *bla*$_{\text{CTX-M-9subgroup}}$ (561bp) at different concentration of Multi CTX-M-G1, Multi CTX-M-G2 and Multi CTX-M-G9 primers-pair. Lanes: 1, 0.15, 0.1 and 0.2 pmol/µl, respectively; 2, 0.3, 0.2 and 0.4 pmol/µl, respectively; 3, 0.4, 0.3 and 0.4 pmol/µl, respectively; 4, 0.8, 0.8 and 0.8 pmol/µl, respectively; 5, negative control; M, 100bp DNA marker

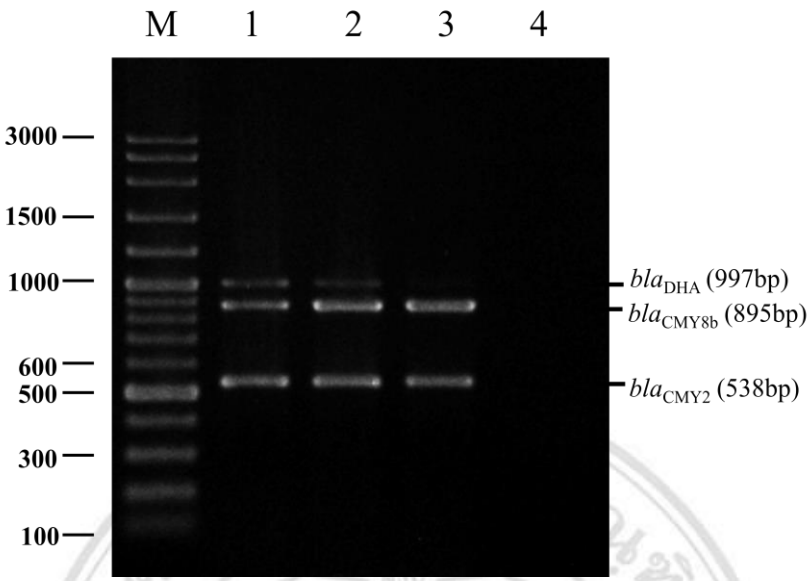

Figure 5.9 Multiplex III: Multiplex PCR products of *bla*$_{CMY1}$ (895 bp), *bla*$_{CMY2}$ (538bp) and *bla*$_{DHA}$ (997bp) at different concentration of MultiCD-CMY1, MultiCD-CMY2 and MultiCD-DHA primers-pair. Lanes: 1, 0.1, 0.1 and 0.25 pmol/µl, respectively; 2, 0.2, 0.2 and 0.5 pmol/µl, respectively; 3, 0.4, 0.4 and 1 pmol/µl, respectively; 4, negative control; M, 100bp DNA marker

5.2.2 To determine ESBL and AmpC β-lactamase genes in β-lactamase-producing *E. coli* strains

One hundred and eighty two clinical isolates were detected ESBL genes by using Multiplex I (*bla*$_{TEM}$ and *bla*$_{SHV}$) and Multiplex II (*bla*$_{CTX-M}$). Representative isolates with ESBL genes are show in Figure 5.10 and 5.11. All cefoxitin resistance isolates were detected with Amp C β-lactamase gene and 10 isolates were found *bla*$_{CMY-2}$ (Figure 5.12). ESBL genes were detected in 172 isolates (94.5%), AmpC β-lactamase gene was observed in 1 isolate (0.5%) and 9 isolates (4.9%) were found both ESBL and AmpC β-lactamase genes. The distribution of the genotypes detected among 181 ESBL producing isolates were *bla*$_{TEM}$ 150 (82.9%), *bla*$_{SHV}$ 20 (11%) and *bla*$_{CTX-M}$ 146 (80.7%). *bla*$_{CTX-M}$ comprise *bla*$_{CTX-M-1subgroup}$ 90 and *bla*$_{CTX-M-9\ subgroup}$ 64 isolates. None of the *bla*$_{CTX-M-subgroup2}$ was present in all isolates. However, in this study, 63 isolates showed unexpected band approximately 450 bps that indicated Multi CTX-M-G2 primers were cross-reactivity with other *bla*$_{CTX-M}$ genes. Some isolates that found unexpected

amplification product were proved by nucleotide sequencing. The results were found unexpected nucleotide sequence similar to *bla*$_{\text{CTX-M-123}}$ gene. Nevertheless, direct sequencing was not enough therefore phylogenetic tool was used for further analysis. The phylogenetic analysis based on deduced nucleotide sequence similarities of CTX-M ESBL suggested that CTX-M-123 is might be a member of the CTX-M-9 subgroup (Figure 5.13).

The genotypes occurred singularly in 45 (24.7%) of the isolates and in several gene combination among the remaining 137 (75.3%). *bla*$_{\text{TEM}}$+ *bla*$_{\text{CTX-M-1}}$ was the most frequent gene combination found in 64 (35.2%) of the isolates followed by *bla*$_{\text{TEM}}$ + *bla*$_{\text{CTX-M-9subgroup}}$ in 40 (22.0%) and *bla*$_{\text{TEM}}$ + *bla*$_{\text{SHV}}$ in 12 (6.6%) (Table 5.1). All isolates were characterized the genotype by multiplex PCR and MIC by agar dilution for cefotaxime and ceftazidime. They are show in Table 5.2. Nucleotide sequencing and phylogenetic analysis were used to identified and confirm ESBL and and AmpC β-lactamase genes. The results showed multiplex PCR could discriminate type of gene (Figure 5.14).

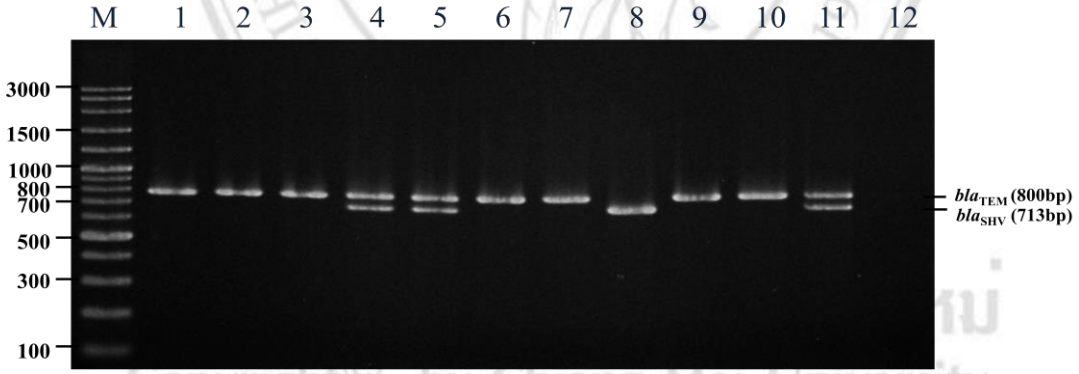

Figure 5.10 PCR products of *bla*$_{\text{TEM}}$ (800bp) and *bla*$_{\text{SHV}}$ (713bp) generated with Multiplex I at optimal condition. Lanes: 1, CM0453-003; 2, CM0453-004; 3, CM0453-006; 4, CM0453-007; 5, CM0453-008; 6, CM0453-010; 7, CM0453-011; 8, CM0453-012; 9, CM0453-014; 10, CM0453-015; 11, Genomic DNA of TEM and SHV strains were used as positive control; 12, negative control; M showed 100bp DNA marker

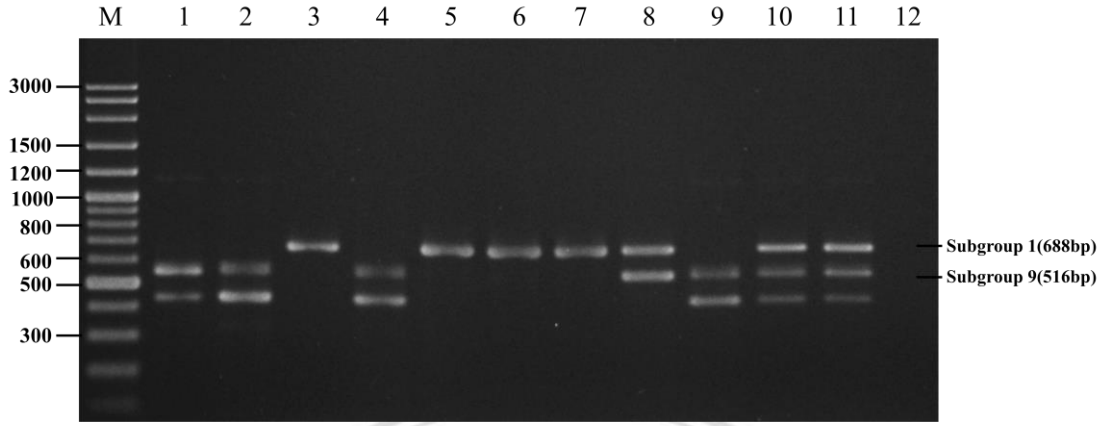

Figure 5.11 PCR products of *bla*$_{\text{CTX-M-1subgroup}}$ (688bp) and *bla*$_{\text{CTX-M-9subgroup}}$ (561bp) generated with Multiplex II at optimal conditions. Lanes: 1, CM0453-003; 2, CM0453-004; 3, CM0453-006; 4, CM0453-007; 5, CM0453-009; 6, CM0453-009; 7, CM0453-012; 8, CM0453-018; 9, CM0453-034; 10, CM0453-039; 11, Genomic DNA of CTX-M-14 and CTX-M-15 strains were used as positive control; 12, negative control; M showed 100bp DNA marker.

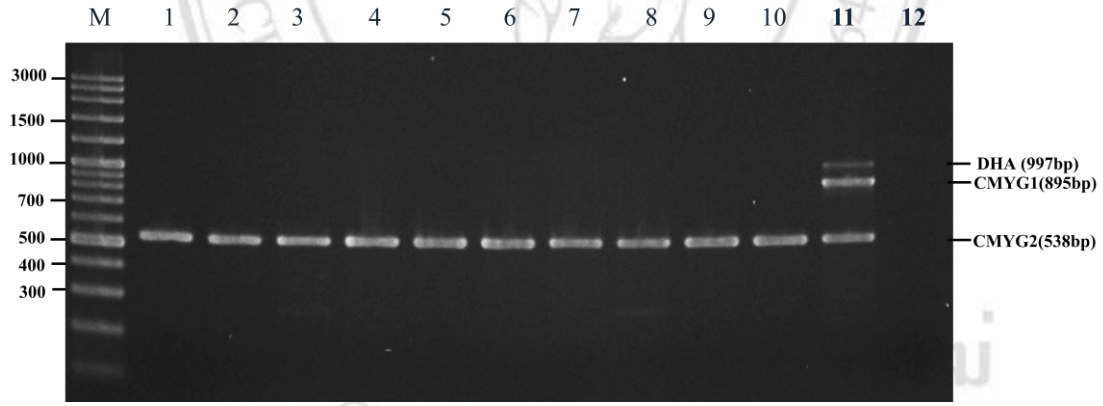

Figure 5.12 PCR products of *bla*$_{\text{CMY8b}}$ (895bp), *bla*$_{\text{CMY2}}$ (538bp) and *bla*$_{\text{DHA}}$ (997bp) generated with Multiplex III at optimal condition. Lanes: 1, CM0453-005; 2, CM0453-010; 3, CM0453-011; 4, CM0453-041; 5, CM0453-042; 6, CM0453-053; 7, CM0453-106; 8, CM0453-141; 9, CM0453-143; 10, CM0553-163; 11, Genomic DNA of CMY8b, CMY2 and DHA-1 strains were used as positive control; 12, negative control; M showed 100bp DNA marker.

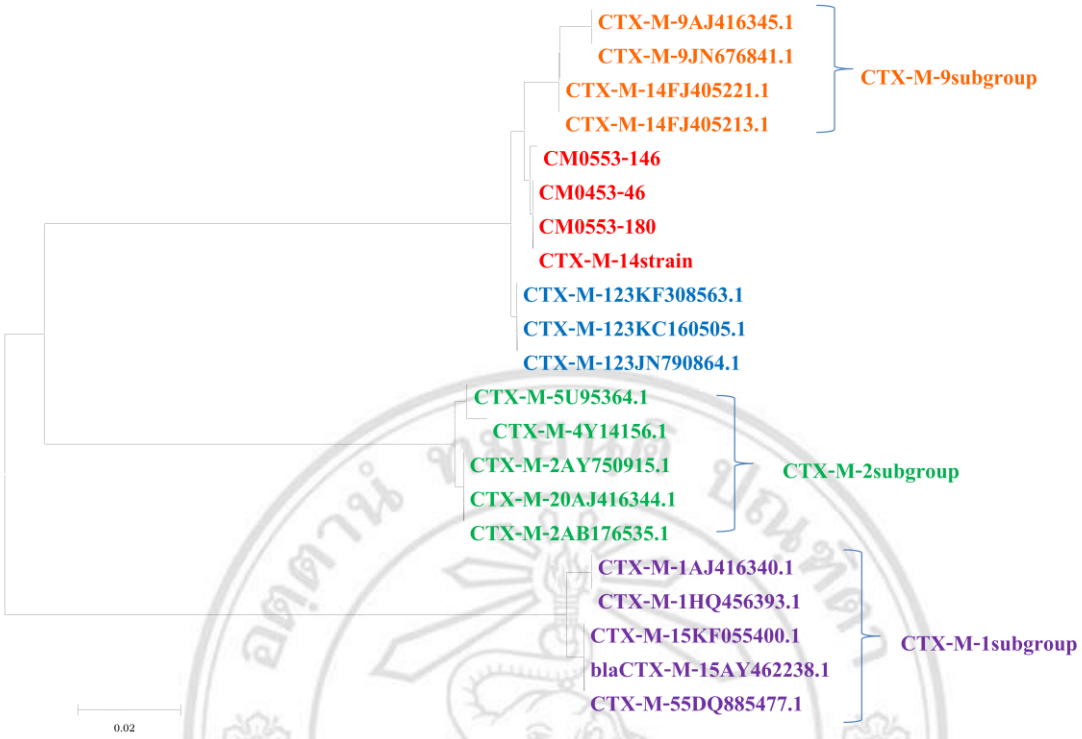

Figure 5.13 Multiple nucleotide sequence alignments were obtained using phylogenetic analysis with *bla* gene of unexpected amplification product (red) compare with other CTX-M-subgroup from the nucleotide database (orange, blue, green and purple) .

Table 5.1 Distribution of ESBL and AmpC β-lactamase genes among 182 clinical isolates

Gene types	Number of isolates	Percentage
TEM	15	8.2%
SHV	3	1.6%
CTX-M-1subgroup	13	7.1%
CTX-M-9subgroup	13	7.1%
CMY-2	1	0.5%
TEM+SHV	12	6.6%
TEM+ CTX-M-1subgroup	64	35.2%
TEM+ CTX-M-9subgroup	40	22.0%
TEM+CMY-2	5	2.7%
TEM+ CTX-M-1subgroup+ CTX-M-9subgroup	7	3.8%
TEM+ CTX-M-1subgroup+CMY-2	3	1.6%
TEM+SHV+ CTX-M-9subgroup	2	1.1%
TEM+ CTX-M-9subgroup+CMY-2	1	0.5%
TEM+SHV+ CTX-M-1subgroup	1	0.5%
SHV+ CTX-M-1subgroup	1	0.5%
SHV+ CTX-M-1subgroup+CTX-M-9subgroup	1	0.5%
Total	182	100%

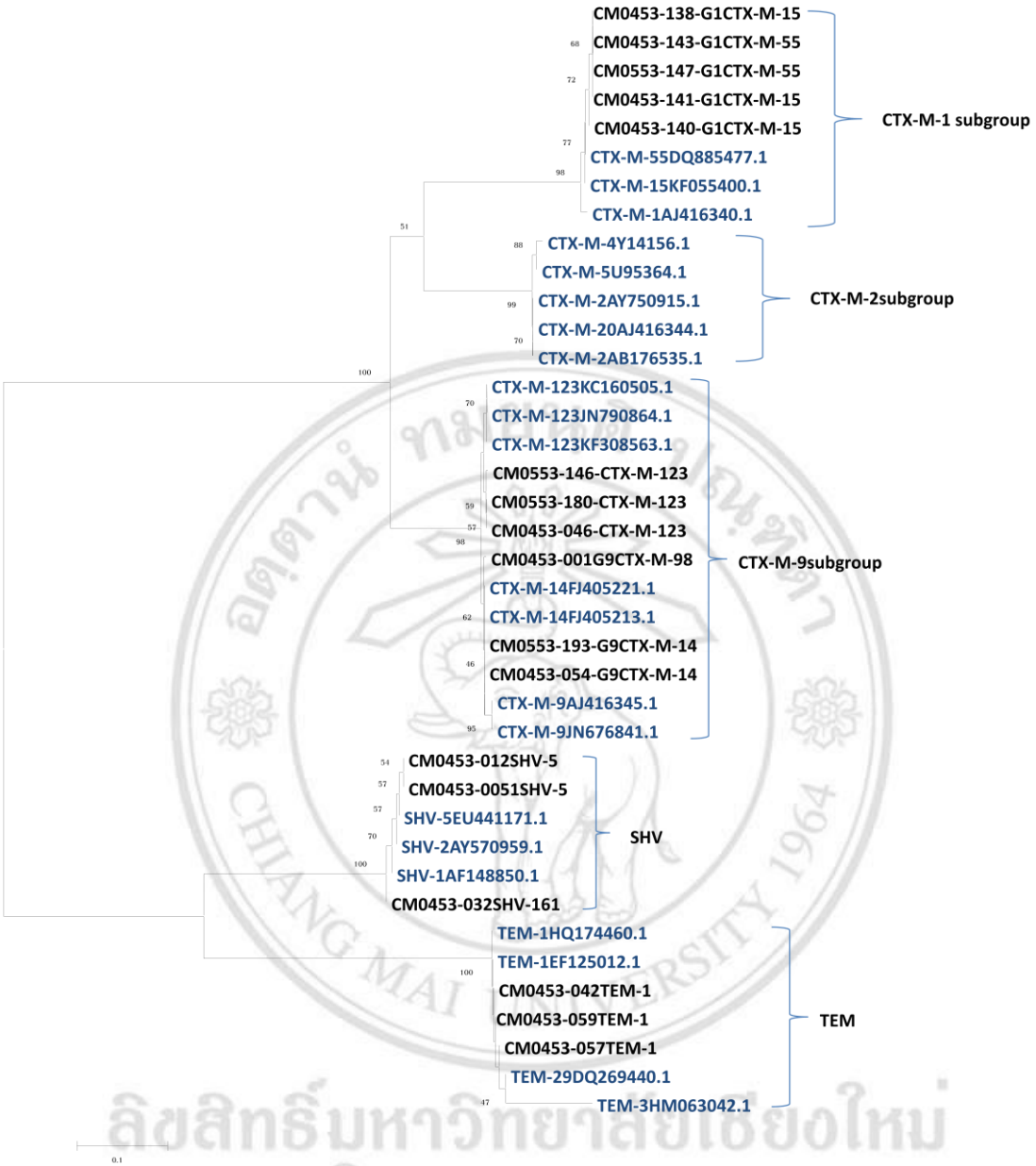

Figure 5.14 multiple nucleotide sequence alignments were obtained using phylogenetic analysis of *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{CTX-M-subgroup}}$ that are in black compare with *bla* gene from nucleotide database that are in blue.

Table 5.2 MIC and molecular characterization of *bla* genes of clinical isolates.

CM no.	MIC(µg/ml)		β-Lactamase	
	Cefotaxime	Ceftazidime	ESBL	AmpC
CM0453-001	64	4	CTX-M-G9	
CM0453-002	128	4	CTX-M-G9	
CM0453-003	8	1	TEM	
			CTX-M-G9	
CM0453-004	64	1	TEM	
			CTX-M-G9	
CM0453-005	8	16		CMY
CM0453-006	512	64	TEM	
			CTX-M-G1	
CM0453-007	128	16	TEM	
			SHV	
			CTX-M-G9	
CM0453-008	8	8	TEM	
			SHV	
CM0453-009	256	128	TEM	
			CTX-M-G1	
CM0453-010	16	32	TEM	CMY
CM0453-011	8	8	TEM	CMY
CM0453-012	256	16	SHV	
			CTX-M-G1	
CM0453-013	64	8	TEM	
CM0453-014	256	16	TEM	
			CTX-M-G1	
CM0453-015	256	32	TEM	
			CTX-M-G1	
CM0453-017	128	1024	TEM	
CM0453-018	512	64	TEM	
			CTX-M-G1	
			CTX-M-G9	
CM0453-019	512	32	TEM	
			CTX-M-G1	
CM0453-021	512	32	TEM	
			CTX-M-G1	
CM0453-022	512	32	TEM	
			CTX-M-G1	
CM0453-024	128	32	TEM	
			CTX-M-G1	
CM0453-025	128	512	TEM	

Table 5.2 (continued)

CM no.	MIC(µg/ml)		β-Lactamase	
	Cefotaxime	Ceftazidime	ESBL	AmpC
CM0453-026	512	32	CTX-M-G1	
CM0453-027	256	64	TEM	
			CTX-M-G1	
CM0453-028	64	4	TEM	
CM0453-029	512	128	TEM	
			CTX-M-G1	
CM0453-030	512	64	TEM	
			CTX-M-G1	
CM0453-031	8	64	TEM	
CM0453-032	1024	128	SHV	
CM0453-033	1	64	SHV	
CM0453-034	32	2	TEM	
			CTX-M-G9	
CM0453-036	64	>1024	TEM	
CM0453-037	16	1204	TEM	
			CTX-M-G9	
CM0453-038	64	4	TEM	
			SHV	
			CTX-M-G9	
CM0453-039	512	64	TEM	
			CTX-M-G1	
			CTX-M-G9	
CM0453-041	256	64	TEM	CMY
			CTX-M-G1	
CM0453-042	16	64	TEM	CMY
CM0453-043	256	64	TEM	
			SHV	
CM0453-044	256	8	TEM	
			SHV	
CM0453-046	512	64	TEM	
			CTX-M-G1	
			CTX-M-G9	
CM0453-047	512	64	TEM	
			CTX-M-G1	
CM0453-048	128	8	TEM	
			CTX-M-G9	
CM0453-049	256	16	TEM	

Table 5.2 (continued)

CM no.	MIC(µg/ml)		β-Lactamase	
	Cefotaxime	Ceftazidime	ESBL	AmpC
CM0453-050	128	16	TEM	
			CTX-M-G1	
CM0453-051	128	16	TEM	
			SHV	
			CTX-M-G1	
CM0453-052	512	256	TEM	
			CTX-M-G1	
CM0453-053	16	64	TEM	CMY
CM0453-054	64	1	TEM	
			CTX-M-G9	
CM0453-056	256	4	CTX-M-G9	
CM0453-057	256	16	TEM	
CM0453-058	128	128	TEM	
			CTX-M-G1	
CM0453-059	128	128	TEM	
			CTX-M-G1	
CM0453-060	256	64	CTX-M-G1	
CM0453-061	512	64	CTX-M-G1	
CM0453-062	512	32	TEM	
			CTX-M-G1	
CM0453-064	512	32	CTX-M-G1	
CM0453-065	256	16	TEM	
			CTX-M-G1	
CM0453-066	256	16	TEM	
			CTX-M-G1	
CM0453-067	256	32	CTX-M-G1	
CM0453-068	256	64	CTX-M-G1	
CM0453-072	32	1	CTX-M-G9	
CM0453-073	64	2	TEM	
			CTX-M-G9	
CM0453-074	128	64	TEM	
			CTX-M-G1	
CM0453-075	4	32	TEM	
			SHV	
CM0453-076	256	64	TEM	
			CTX-M-G1	

Table 5.2 (continued)

CM no.	MIC(µg/ml)		β-Lactamase	
	Cefotaxime	Ceftazidime	ESBL	AmpC
CM0453-077	256	32	TEM	
			CTX-M-G1	
CM0453-078	128	1	CTX-M-G9	
CM0453-079	16	1	TEM	
			CTX-M-G9	
CM0453-080	8	64	SHV	
CM0453-081	512	16	TEM	
			CTX-M-G1	
CM0453-082	1024	128	TEM	
			CTX-M-G1	
CM0453-083	128	16	TEM	
CM0453-084	256	64	TEM	
			CTX-M-G1	
CM0453-085	128	64	TEM	
			CTX-M-G1	
CM0453-086	256	>1024	TEM	
CM0453-087	64	8	TEM	
			CTX-M-G9	
CM0453-088	64	64	TEM	
			CTX-M-G1	
CM0453-089	256	16	TEM	
			CTX-M-G1	
CM0453-090	512	64	TEM	
			CTX-M-G1	
CM0453-091	64	1	TEM	
			CTX-M-G9	
CM0453-092	128	2	CTX-M-G9	
CM0453-094	32	2	TEM	
			CTX-M-G9	
CM0453-095	128	8	TEM	
			CTX-M-G1	
			CTX-M-G9	
CM0453-096	64	4	TEM	
			CTX-M-G9	
CM0453-097	128	32	TEM	
			CTX-M-G9	

Table 5.2 (continued)

CM no.	MIC(μg/ml)		β-Lactamase	
	Cefotaxime	Ceftazidime	ESBL	AmpC
CM0453-098	64	2	TEM	
			CTX-M-G9	
CM0453-099	128	32	TEM	
			CTX-M-G9	
CM0453-101	512	16	TEM	
			CTX-M-G1	
CM0453-102	256	16	TEM	
			CTX-M-G1	
CM0453-103	256	8	TEM	
			CTX-M-G1	
CM0453-104	64	8	CTX-M-G9	
CM0453-105	1024	128	TEM	
CM0453-106	32	32	TEM	CMY
			CTX-M-G9	
CM0453-107	256	>1024	TEM	
			SHV	
CM0453-108	64	2	TEM	
			CTX-M-G9	
CM0453-109	64	4	TEM	
CM0453-110	256	>1024	TEM	
			SHV	
CM0453-111	32	2	TEM	
			CTX-M-G9	
CM0453-112	256	8	TEM	
			CTX-M-G1	
CM0453-113	256	>1024	TEM	
			SHV	
CM0453-114	512	32	TEM	
			CTX-M-G1	
CM0453-115	256	32	TEM	
CM0453-116	128	16	TEM	
			CTX-M-G1	
CM0453-117	128	32	TEM	
CM0453-118	512	64	TEM	
			CTX-M-G1	
CM0453-119	128	>1024	TEM	
			SHV	

Table 5.2 (continued)

CM no.	MIC(µg/ml)		β-Lactamase	
	Cefotaxime	Ceftazidime	ESBL	AmpC
CM0453-120	512	64	TEM CTX-M-G1	
CM0453-121	128	32	TEM CTX-M-G1	
CM0453-122	128	8	TEM CTX-M-G9	
CM0453-123	256	32	TEM CTX-M-G1	
CM0453-124	256	4	TEM CTX-M-G1 CTX-M-G9	
CM0453-125	512	64	TEM CTX-M-G1	
CM0453-126	512	32	TEM CTX-M-G1	
CM0453-127	128	32	TEM CTX-M-G1	
CM0453-128	128	>1024	TEM SHV	
CM0453-129	512	32	TEM CTX-M-G9	
CM0453-130	512	16	CTX-M-G1	
CM0453-131	256	>1024	TEM SHV	
CM0453-132	1024	16	CTX-M-G1	
CM0453-133	128	4	TEM CTX-M-G9	
CM0453-134	256	32	TEM CTX-M-G1	
CM0453-135	64	1	TEM CTX-M-G1	
CM0453-136	256	16	TEM CTX-M-G1	
CM0453-137	128	32	CTX-M-G1	
CM0453-138	64	2	CTX-M-G9	
CM0453-139	256	32	TEM CTX-M-G1	
CM0453-140	256	8	TEM CTX-M-G1	

Table 5.2 (continued)

CM no.	MIC(µg/ml)		β-Lactamase	
	Cefotaxime	Ceftazidime	ESBL	AmpC
CM0453-141	1024	128	TEM CTX-M-G1	CMY
CM0453-142	64	4	CTX-M-G9	
CM0453-143	512	128	TEM CTX-M-G1	CMY
CM0453-144	64	4	CTX-M-G9	
CM0553-145	128	4	TEM SHV	
CM0553-146	512	128	CTX-M-G9	
CM0553-147	32	2	TEM CTX-M-G1	
CM0553-148	512	4	TEM CTX-M-G9	
CM0553-149	128	8	TEM CTX-M-G9	
CM0553-150	128	8	TEM CTX-M-G9	
CM0553-151	512	32	TEM CTX-M-G1 CTX-M-G9	
CM0553-152	512	32	TEM CTX-M-G9	
CM0553-153	512	128	CTX-M-G1	
CM0553-154	256	32	TEM CTX-M-G1	
CM0553-155	64	2	TEM CTX-M-G9	
CM0553-156	256	32	TEM CTX-M-G9	
CM0553-157	1024	128	TEM CTX-M-G1	
CM0553-158	512	32	SHV CTX-M-G1 CTX-M-G9	
CM0553-159	32	2	TEM CTX-M-G9	

Table 5.2 (continued)

CM no.	MIC(µg/ml)		β-Lactamase	
	Cefotaxime	Ceftazidime	ESBL	AmpC
CM0553-160	32	1	TEM CTX-M-G9	
CM0553-163	32	64	TEM CTX-M-G9	CMY
CM0553-164	256	16	TEM CTX-M-G1	
CM0553-165	>1024	256	CTX-M-G9	
CM0553-166	32	1	TEM CTX-M-G9	
CM0553-167	512	32	TEM	
CM0553-168	128	32	TEM CTX-M-G1	
CM0553-169	256	32	TEM CTX-M-G9	
CM0553-170	512	128	TEM CTX-M-G1	
CM0553-172	64	8	TEM CTX-M-G9	
CM0553-173	32	1	TEM CTX-M-G9	
CM0553-174	32	1	TEM CTX-M-G9	
CM0553-175	64	8	TEM CTX-M-G9	
CM0553-176	256	32	TEM	
CM0553-177	128	16	TEM CTX-M-G1	
CM0553-178	64	1	TEM CTX-M-G9	
CM0553-179	128	2	TEM SHV	
CM0553-180	32	1	CTX-M-G9	
CM0553-181	256	32	TEM CTX-M-G1	
CM0553-182	128	32	TEM CTX-M-G1	
CM0553-183	256	128	TEM CTX-M-G1	

Table 5.2 (continued)

CM no.	MIC(µg/ml)		β-Lactamase	
	Cefotaxime	Ceftazidime	ESBL	AmpC
CM0553-184	128	64	CTX-M-G1	
CM0553-185	64	2	TEM	
			CTX-M-G9	
CM0553-186	64	8	TEM	
			CTX-M-G9	
CM0553-187	512	64	CTX-M-G1	
CM0553-189	1024	64	TEM	
			CTX-M-G1	
CM0553-190	512	32	TEM	
			CTX-M-G1	
CM0553-191	128	2	TEM	
			CTX-M-G9	
CM0553-192	64	32	TEM	
			CTX-M-G9	
CM0553-193	128	256	TEM	
			CTX-M-G1	
			CTX-M-G9	
CM0553-195	256	4	TEM	
			CTX-M-G1	
CM0553-196	1024	256	TEM	
			CTX-M-G1	
CM0553-197	256	16	TEM	
			CTX-M-G1	
CM0553-198	128	32	TEM	
			CTX-M-G1	
CM0553-199	512	32	TEM	
			CTX-M-G1	
CM0553-200	256	64	CTX-M-G1	

(CTX-M-G1: CTX-M-1subgroup, CTX-M-G9: CTX-M-9subgroup)

5.3 Correlation between distributions of the MICs for ESBL-producing *E. coli* with the presence of ESBL genes

Because of each type of ESBL enzyme are different efficiently to hydrolyze extended-spectrum β-lactamase antibiotic. Therefore, the different types of ESBL or combined types of different ESBL genes might be correlated with the level of the phenotypic resistance to antimicrobial agents. In this study, the correlation of phenotypic resistance to antibiotic and ESBL gene was analyzed by using SPSS. The levels of resistance to cefotaxime were significantly correlated with the presence of $bla_{\text{CTX-M-1subgroup}}$ (p-value = 0.000) (high resistance; MIC ≥ 128 µg/ml) and $bla_{\text{CTX-M-9 subgroup}}$ (p-value = 0.000) (Figure 5.15) and both genes were found only in cefotaxime-resistant isolates. The levels of resistance to cefotaxime did not correlate with bla_{TEM} (p-value = 0.509) and bla_{SHV} (p-value = 0.327). The resistance to ceftazidime was significantly correlated with the presence of bla_{SHV} (p-value = 0.034) and $bla_{\text{CTX-M-1subgroup}}$ (p-value = 0.037) but it did not correlated with presence $bla_{\text{CTX-M-9 subgroup}}$ (p-value = 0.511) and bla_{TEM} (p-value = 0.456) (Figure 5.16). Interestingly, isolates carrying the CTX-M-1 subgroup significant reduced susceptibilities to cefotaxime and ceftazidime. All PCR products were submitted to direct sequencing. The analysis was found $bla_{\text{CTX-M-55}}$ and $bla_{\text{CTX-M-15}}$ at 47 and 43 isolates, respectively. However, the presence of $bla_{\text{CTX-M-55}}$ or $bla_{\text{CTX-M-15}}$ not correlated with the level of antimicrobial resistance.

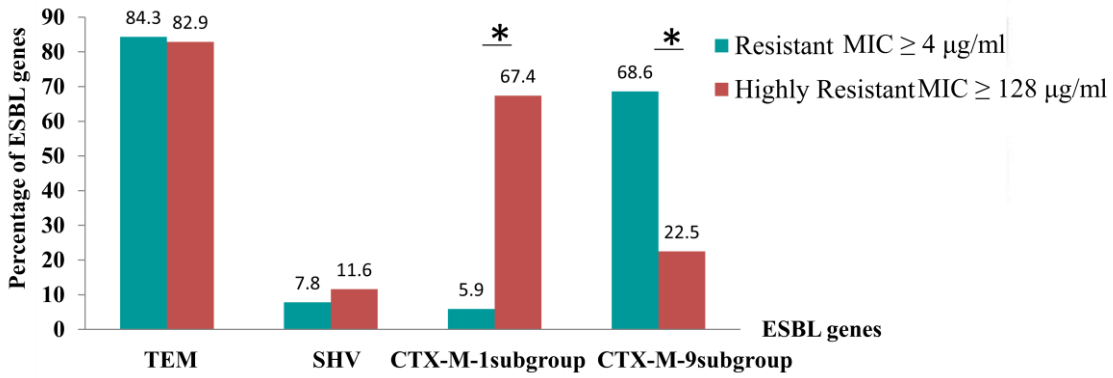

Figure 5.15the correlation between the levels of resistance to cefotaxime in ESBL-producing *E. coli* and presence of ESBL genes (resistant MIC ≥4 µg/ml and highly resistant MIC MIC ≥ 128 µg/ml, n=182 isolates,*P<0.05 with Fisher' exact test).

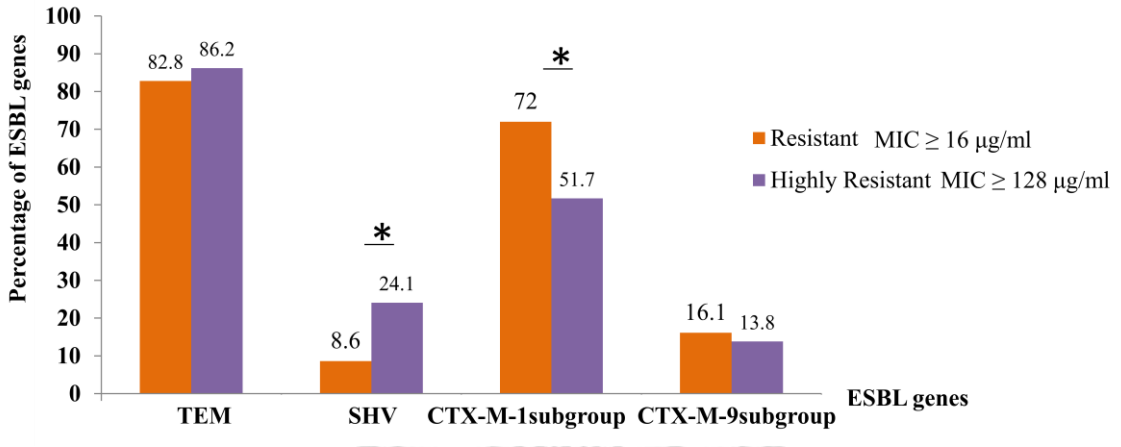

Figure 5.16 the correlation between the levels of resistance to ceftazidime in ESBL-producing *E. coli* and presence of ESBL genes (resistant MIC ≥8 µg/ml and highly resistant MIC MIC ≥ 128 µg/ml, n=182 isolates, **P*<0.05 with Fisher' exact test).

The number of combined types of different ESBL was studied for correlation with level of resistance to cefotaxime and ceftazidime. The single type of ESBL gene exhibited a high resistance to cefotaxime and ceftazidime comparable to combined types of different ESBL genes. For example, some isolates have single *bla* gene ($bla_{\text{CTX-M-1subgroup}}$) and are resistance to cefotaxime (MIC_{90} = 512 ug/ml) similar to other isolates which have three *bla* genes ($bla_{\text{CTX-M-1subgroup}}$ combine with $bla_{\text{CTX-M-9subgroup}}$ and bla_{TEM}) (Figure 5.17). Therefore, levels of resistance to cefotaxime and ceftazidime did not depend on the accumulating number of genes but related to type of ESBL gene.

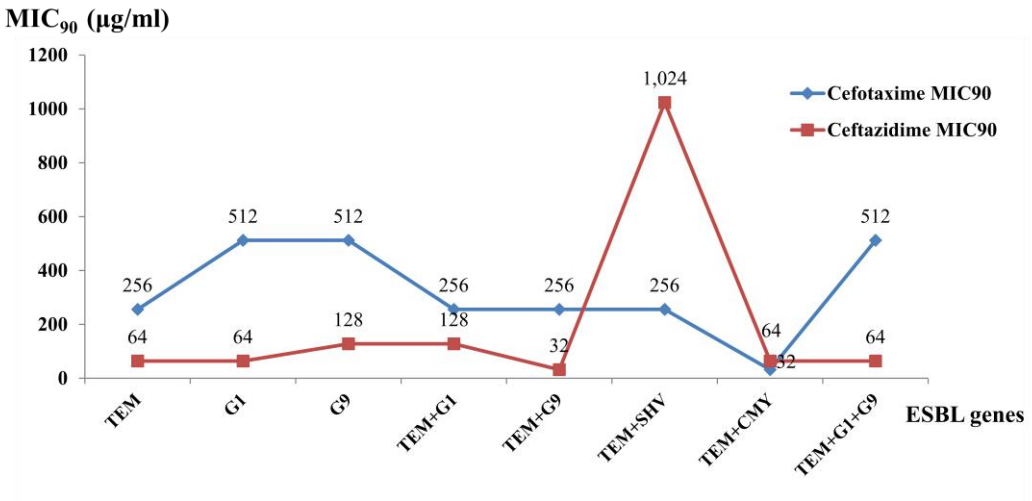

Figure 5.17 correlation between MIC_{90} and single and combined type among different and combined of ESBL genes (G1: CTX-M1subgroup, G9: CTX-M9subgroup).

5.4 Correlation between distributions of the MICs for AmpC β-lactamase-producing *E. coli* with the presence of AmpC β-lactamase genes

Because, in this study were found small number of isolates (5.5%) carried AmpC β-lactamase genes and all of isolates were *bla*$_{CMY-2.}$ Therefore, our study could not discern such findings to the correlation between the resistance to cefotaxime and ceftazidime and the presence of AmpC β-lactamase genes. Moreover, further analysis of isolates including cephamycin and carbapenem hydrolysis assay is necessary to verify these effects. However, the presence of *bla*$_{CMY-2}$ together with ESBL genes also did not increase MICs.