Genotyping of β-Lactamase (*bla*$_{CTX-M}$, *bla*$_{OXA}$, *bla*$_{SHV}$, and *bla*$_{TEM}$) genes from Extended-spectrum β-Lactamase-producing Enterobacteriaceae Clinical Isolates of Ospital ng Maynila Memorial Medical Center, Philippines

Joel C. Cornista[1], Erika R. de Jesus[2], Anna Patrizia B. Pacheco[2], Ramon Arvin Noriel B. Santos[2], Ronald Cabudoy[3], and Marilen P. Balolong*[1]

[1]Department of Biology, College of Arts and Sciences, University of the Philippines Manila, Ermita, Manila 1000 Philippines
[2]Department of Science, College of Arts and Sciences, Miriam College, Katipunan Avenue, Loyola Heights, Quezon City 1108 Philippines
[3]Department of Pathology, Ospital ng Maynila, Quirino Ave, corner Roxas Blvd., Malate, Manila 1004 Philippines

This paper reports the presence of extended-spectrum β-lactamase (ESBL)-producing bacteria exhibiting multiple *bla* genes from sputum, endotracheal aspartate, urine, corneal specimen, wound discharge, breastmilk, and abscess from infected patients of Ospital ng Maynila Memorial Medical Center. About 55 out of 100 clinical isolates were considered as putative ESBL-producers being resistant to more than one of the β-lactam antibiotics tested. They were further identified as *Enterobacter aerogenes*, *Enterobacter hafnia*, *Enterobacter spp*., *Enterobacter cloacae*, *Klebsiella spp*., *Klebsiella pneumoniae*, *Klebsiella rhinoscleromatis*, *Klebsiella cloacae*, *Klebsiella ozaenae*, and *Escherichia coli*. Only 40% of the isolates were confirmed for ESBL-production through the Phenotypic Disk Diffusion Test (PCDDT). The type of β-lactamase genes carried by these ESBL-producers was also determined by amplification of the *bla*$_{CTX-M}$, *bla*$_{OXA}$, *bla*$_{SHV}$, and *bla*$_{TEM}$ genes by Polymerase Chain Reaction. The *bla*$_{CTX-M}$ gene was the most predominant bla gene followed by the *bla*$_{OXA}$ gene which was detected in 53/55 (96.36%) of the isolates. On the other hand, 44/55 (80%) of the isolates possessed the *bla*$_{SHV}$ gene while 38/55 (69.1%) carried the *bla*$_{TEM}$ gene. The presence of multiple *bla* genes within the same strain with 35 out of 55 (63.6%) possessing all the four genes tested were also observed. Ten (18.18%) of the strains possessed three of the β-lactamase genes and another 10 harboured at least two genes. Interestingly, bla genes were also detected even for isolates that were negative for the PCDDT indicating that these genes might be present but the β-lactamase enzyme might not have been actively expressed by the bacteria.

KEYWORDS

bla$_{CTX-M}$, *bla*$_{OXA-1}$, *bla*$_{SHV}$, *bla*$_{TEM}$, extended-spectrum β-lactamase, PCDDT

*Corresponding author
Email Address: mpbalolong@up.edu.ph
Date received: March 15, 2020
Date revised: August 26, 2020
Date accepted: October 20, 2020

INTRODUCTION

Extended-spectrum β-lactamase (ESBL) producing bacteria are commonly found in clinical isolates particularly among Enterobacteriaceae and its prevalence has been reported to continuously increase worldwide. ESBL-producing Enterobacteriaceae cause diseases and infections such as septicaemia, urinary tract infection, pneumonia, wound infections, meningitis, gastroenteritis, and sporadic outbreaks (Tham 2012). Infections caused by ESBL-positive organisms often involve immunocompromised patients, making them difficult to eradicate in high-risk wards, such as intensive care units (Spanu et al. 2002). These antibiotic resistance within Enterobacteriaceae is often due to the production of the extended-spectrum β-lactamase enzymes encoded by the *bla* gene either in the chromosome or plasmid (Schmitt et al. 2007). These enzymes efficiently catalyze the irreversible hydrolysis of beta-lactam drugs such as penicillins and cephalosporins. These ESBLs also exhibit effective hydrolyzation of first-, second- and third generation cephalosporins, and monobactams (Bradford 2001). Although ESBLs were initially predominant in *Klebsiella pneumoniae* and *E. coli* strains, recent studies have shown that other genera of the Enterobacteriaceae family were also found to possessed these enzymes (Ojdana et al. 2014). The reduction of therapeutic possibilities as a result of increasing resistance of these bacteria to antibiotics often caused difficulty in managing infections within hospital settings.

The disk diffusion method such as the PCDDT is considered as standard methods for detecting ESBLs (Modi et al. 2012). This test uses ceftazidime which is a semisynthetic, broad spectrum, beta-lactam antibiotic and has activity against Gram positive and Gram negative organisms especially to *Pseudomonas* sp. and Enterobacteriaceae. Its bactericidal activity results from the inhibition of cell wall synthesis via affinity for penicillin-binding proteins (PBPs). The test also uses cefotaxime that possess a broad spectrum activity against Gram positive and Gram negative bacteria except for *Pseudomonas* sp. and also inhibits the bacterial cell wall biosynthesis via affinity for penicillin-binding proteins (PBPs). Meanwhile, clavulanic acid which is a potent β-lactamase suicide inhibitor is successfully being used in combination to existing β-lactam antibiotics (Paradkar, 2013). This is because a wide variety of beta-lactamases which are commonly found in microorganisms resistant to penicillins and cephalosporins are being inhibited by clavulanic acid. Thus, binding and irreversible inhibition of the beta-lactamase results in a restoration of the antimicrobial activity of beta-lactam antibiotics against lactamase-secreting-resistant bacteria as shown by the increase in the zone of inhibition.

To date, several genetic subtypes of β-lactamase genes have been reported which has already exceeded more than 200 variants (Varkey et al. 2014). But the $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} and bla_{OXA} genes have been reported to be the most common types and variants of these genes formed new dominant ESBLs (Barguigua et al. 2011). These β-lactamase enzymes are commonly classified according to molecular characteristics or functional. The molecular classification divides β-lactamases into four major classes (A–D) based on amino acid similarity (Ambler et al. 1991). On the other hand, the functional classification groups β-lactamases according to similarities in substrate or inhibitor profile (Bush et al. 1995). However, a more updated functional classification places cephalosporinases in group 1, serine β-lactamases in group 2, and the metallo-β-lactamases in group 3 (Bush and Jacoby 2010). Since these *bla* genes encoding antibiotic resistance are often placed on transferable elements such as plasmids or transposons, horizontal gene transfer of antibiotic resistance among bacterial strains are easily facilitated by the localization of the *bla* genes (Ojdana et al. 2014).

The prevalence of extended-spectrum β-lactamase among Enterobacteriaceae have been reported to vary worldwide. In the United States, 26,000 (19%) cases of healthcare associated infections are caused by ESBL-producing Enterobacteriaceae while in Europe, a prevalence of 85–100% ESBL-producing *E. coli* and *K. pneumoniae* have been reported (Lota and Latorre 2013). In the Asia Pacific region however, the prevalence of 42.2% and 35.8% ESBL-positive *E. coli* and *Klebseilla* spp., respectively have been recorded (Hawser et al. 2009). In the Philippines, there are very limited studies of the prevalence of ESBL-producing Enterobacteriaceae. In a study of Bomasang and Mendoza (2003), the prevalence of ESBL-positive clinical isolates of Enterobacteriaceae from the Philippine General Hospital (PGH) was reported to be 29.9%. Besides, prevalence of ESBL-producing *E. coli* and *Klebsiella* spp. in a tertiary hospital in Makati City was shown to be 20.1% **(**Villanueva et al. 2003) while the prevalence of ESBL-producing Enterobacteriaceae at Mindanao Sanitarium and a hospital in southern Philippines was reported to be 5.1% (Lucena et al. 2012). A study on extended-spectrum β-lactamase production in clinical isolates of *E. coli* from three hospitals in Luzon showed a prevalence of around 22.6% (Cruz et al. 2014) and the prevalence of ESBL producing Enterobacteriaceae from clinical isolates in a tertiary hospital in Bacolod City was reported to be18.8% (Garcia et al. 2016). The different ESBL prevalence rate in the country have normally been observed according to the region and time of collection of samples. With the increasing prevalence rate of ESBL-caused infections, it was observed that the mortality rate of patients who are infected are also increasing (Melzer & Petersen 2007).

Studies on determining the type of β-lactamase genes present in ESBL-producing Enterobacteriaceae in the Philippines are also very limited. Cabrera and Rodriguez (2009) reported that $bla_{\text{SHV-12}}$ was the most dominant ESBL among clinical isolates of Enterobacteriaceae in the Philippine General Hospital. Moreover, it was also reported that $bla_{\text{CTX-M}}$ gene to be the most predominant extended-spectrum β-lactamases among Enterobacteriaceae in other parts the Philippines (Tian et al. 2010; Lucena et al. 2012; Cruz and Hedreyda 2017). In a more recent study, the $bla_{\text{OXA-1}}$ gene was found to be the most common *bla* gene among *K. pneumoniae* isolated from fomites in four different hospitals in Luzon (Cornista et al. 2019). Changes in the emergence and predominance of different ESBL genes throughout the Philippines prove their wide occurrence in the country.

Because of the increasing prevalence of bacteria with multiple ESBL-genes among Enterobacteriaceae predominantly due to indiscriminate use of antibiotics, this has become a major concern within hospital setting (Paterson & Bonomo 2005). But the complexity and expensiveness of the detection of ESBL production through the practice of routine laboratory susceptibility testing methods implicates why there are only a few studies on the detection of ESBL-producing Enterobacteriaceae in Philippine hospital settings (Lota & Latorre 2014). A close monitoring and surveillance system however, plays a significant role in controlling the spread of ESBL-producing bacteria in the country. But a reliable identification of ESBL-producing organisms in clinical laboratories can be challenging and time-consuming, thus, in the Philippines prevalence of ESBL are likely underestimated. This further strengthens the urgent need of a routine molecular screening for ESBL in the Philippines to provide fast and accurate results. The results of this study therefore, would contribute to the poor baseline data on the prevalence and

Table 1: Summary of the primers used for the amplification of the different β-lactamase genes and the expected amplicon sizes (Dallenne et al. 2010).

Gene	Forward Primer (5'→ 3')	Reverse Primer (5' → 3')	Amplicon size (bp)
bla$_{CTX-M}$	5'-TTAGGAAGTGTG CCGCTGCA-3'	5'- CGATATCGTTGG TGGTGCCAT- 3'	688
bla$_{OXA-1}$	5'-GGCACCAGATTC AACTTTCAAG-3'	5'-GACCCCAAGTTT CCTGTAAGTG-3'	564
bla$_{TEM}$	5'-CATTTCCGTGTC GCCCTTATTC-3'	5'-CGTTCATCCATAG TTGCCTGAC-3'	800
bla$_{SHV}$	5'-AGCCGCTTGAG CAAATTAAAC -3'	5'-ATCCCGCAGATAA ATCACCAC-3'	713

Table 2: Summary of the source and the number of clinical samples and their identities.

Identities	Sputum	ETA	Wound	Urine	Corneal	Breastmilk	Abscess
Klebsiella spp.	3	2	1	-	-	-	-
K. cloacae	-	-	-	-	-	1	-
K. ozaenae	-	-	-	-	-	1	-
K. pneumoniae	10	11	4	2	-	-	1
K. rhinoscleromatis	1	1	-	1	1	-	-
Enterobacter spp.	2	1	2	3	-	-	-
E. hafnia	1	-	-	-	-	-	-
E. cloacae	-	-	1	-	-	-	-
E. aerogenes	-	1	1	-	1	-	-
Escherichia coli	-	-	-	2	-	-	-

*Endotracheal aspirate (ETA)

genotyping of ESBL-producers particularly among members of the family Enterobacteriaceae in the Philippines.

METHODS

Sample Collection

Clinical isolates were obtained from the Bacteriology Section, Department of Pathology, Ospital ng Maynila Memorial Medical Center, Philippines. Approval from the Ethics Committee of both Ospital ng Maynila Memorial Medical Center and Miriam College, Quezon City were first obtained prior to the conduct of the study. Samples were collected from sputum, endotracheal aspartate, urine, corneal specimen, wound discharge, breastmilk, and abscess of infected patients, male and female, ages ranging from a newborn until 87 years old. The sample collection period started from September 2018 until the end of April 2019. Since extended-spectrum β-lactamase (ESBL)-producing bacteria have been commonly reported in members of the family Enterobacteriaceae in which most of the medically important bacteria in hospital infections are found, these group were actively selected in this study. Clinical samples were serially diluted and 100 µl was plated onto Mac Conkey agar and incubated at 37 °C for 24 h. At least three bacterial colonies per clinical sample were randomly selected and purified in Nutrient Agar (NA) plates. These isolates were then initially characterized using standard morphological and biochemical tests based on the Bergey's Manual of Determinative Bacteriology.

Purified bacterial isolates from clinical samples were transported in a thermal cooler to the Microbiology Research Laboratory of Miriam College, Quezon City for the Phenotypic Confirmatory Disk Diffusion Test (PCDDT), molecular characterization and genotyping of ESBL genes. The positive control of ESBL-producing *K. pneumoniae* BUL_ICUVent was isolated from a hospital in Bulacan, Philippines and confirmed to be ESBL-producing by both antimicrobial susceptibility testing and PCDDT. This strain was previously reported to possessed the *bla*$_{CTX-M}$, *bla*$_{OXA}$, and *bla*$_{TEM}$ genes (Cornista et al. 2019). A negative control of non-ESBL-producing *Klebsiella pneumoniae* (KpB32) was obtained from the Culture Collection of the Biology Department of University of the Philippines Manila.

Antimicrobial Susceptibility Testing

A total of 100 different clinical isolates were initially tested for antibiotic susceptibility against ampicillin/sulbactam (SAM), piperacillin/tazobactam (TZP), ceftazidime (CAZ), cefotaxime (CTX), ceftriaxone (CRO), and imipenem (IPM) using standard disk diffusion method. These antibiotics were selected because these are the commonly used beta-lactam antibiotics in hospitals and are recommended for screening of ESBLs (CLSI, 2012). Briefly, two to three distinct colonies from each purified plate were picked up and inoculated into Mueller Hinton Broth (MHB) and incubated at 37 °C for 16–18 h. The bacterial suspension was adjusted to 0.5 McFarland standard with saline solution. The cultures were then swabbed onto Mueller Hinton agar (MHA) plates and disks containing different antibiotics were placed on top of the swabbed plates and incubated overnight at 37 °C. The clearing zones were measured and interpreted based on the guidelines set by the Clinical Laboratory Standards Institute (CLSI, 2012). Bacterial isolates that were resistant to more than one of the different antibiotics tested were considered as ESBL-suspect. Only isolates that were initially determined to be ESBL-positive through AST were further confirmed for ESBL-production through the PCDDT.

Confirmation of ESBL Production

The presence of ESBL enzymes was confirmed by PCDDT as recommended by CLSI (2013). Overnight cultures of the bacterial isolates were grown in MHB and suspensions were adjusted with saline solution to achieve turbidity of 0.5 McFarland standard. Aliquots of the suspensions were swabbed on the surface of MHA plates, covering the entire surface. Disks of ceftazidime (30 µg) and ceftazidime/ clavulanic acid (30/10 µg)

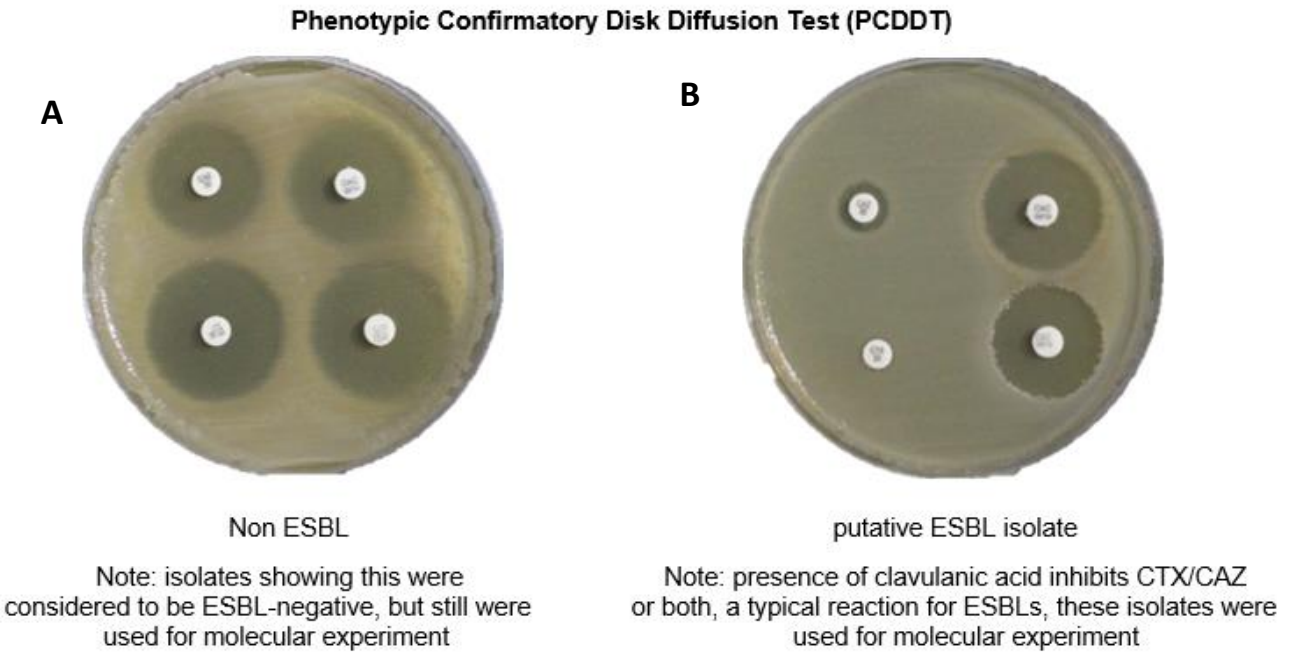

Figure 1: PCDDT of isolates using antibiotic discs containing ceftazidime (CAZ) and ceftazidime/clavulanic acid (CAC) and cefotaxime (CTX) with cefotaxime/clavulanic acid (CEC). (A): show the results for a non-ESBL producing *K. pneumoniae* (B): shows the results for ESBL-producing *Klebsiella spp* showing an increase of at least 5 mm in the diameter of the zone of inhibition produced with antibiotic plus clavulanic acid.

were placed on the upper part of the MHA plate while disks of cefotaxime (30 µg) and cefotaxime/clavulanic acid (30/10 µg) were placed on the lower part. The disks were positioned onto the plate at a distance of 30 mm from each other and incubated for 18 hours at 37 °C. ESBL-production of the different isolates were confirmed when an increase of ≥ 5 mm in the zone diameter was observed for either antimicrobial agent tested in combination with clavulanic acid over when tested alone.

Confirmation of Identity of ESBL-producing isolates

The identity of a representative ESBL-producing Enterobacteriaceae isolates were confirmed by amplification of the *16S rRNA* gene through Polymerase Chain Reaction (PCR). First, the genomic DNA were extracted using the GF-1 Bacterial DNA Extraction Kit (GF-BA-100) (Vivantis, Malaysia) and were used as TEMplates for PCR amplification and16S *rRNA* gene using a Quanta S1-96 Thermocycler. The following 16S *rRNA* forward (5' TGT GGG AAC GGC GAG TCG GAA TAC 3') and 16S *rRNA* reverse (5' GGG CGC AGG GGA TGA AAC TCA AC 3') primers were used (Shahi et al. 2013). The PCR reaction was composed of 25 µl working volume which was made up of 5.5 µl sterile distilled water, 5 µl extracted bacterial DNA, 12.5 µl 2X Taq Master Mix, 1 µl of 10 µM16S *rRNA* gene forward primer and 1 µl of 10 µM 16S *rRNA* gene reverse primer. The maximized PCR conditions used were as follows: initial denaturation at 95°C for 5 min; 35 cycles of denaturation (1 min at 94°C); annealing (20 sec at 59°C); extension (1 min at 72°C); and final extension at 72°C for 5 min. The presence and purity of the amplified fragments were checked by Agarose Gel Electrophoresis (AGE) in 1.5% agarose gel run at 100 V for 30 min. A total of 20 µl of the amplicons (100-150 ng/ µl) from the ESBL-producing Enterobacteriaceae isolates were then sent to Macrogen, Korea for gene sequencing using the Sanger Method. The DNA sequences were then be aligned using Basic Local Alignment Sequencing Tool (BLAST), and the % homologies and possible identities were determined from the PUBMED nucleotide database.

Detection of β-lactamase Genes

Genotyping of the different β-lactamase genes responsible for the ESBL production of the different Enterobacteriaceae isolates were determined by amplification of the *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{OXA}$, genes by PCR. The following sets of primers and the expected amplicon sizes are shown in Table 1.

The total reaction mixture of 25 µl was composed of the following: 5 µl of the extracted genomic DNA, 5.5 µl sterile distilled H_2O, 1µL each of 10 µM forward and reverse primers and 12.5 µl of 2X Taq Master Mix. For the amplification of the *bla*$_{CTX-M}$, and *bla*$_{OXA}$ genes, the following PCR conditions were used: 94 °C for 4 min (initial denaturation), 35 cycles of denaturation at 94 °C for 1 min, 55 °C for 20 sec (annealing), 72 °C for 30 sec (extension) and 72 °C for 5 min (final extension). On the other hand, for the amplification of the *bla*$_{SHV}$ gene, the following PCR conditions were used: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 54.5 °C for 15 sec, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. Lastly, for the detection of *bla*$_{TEM}$ gene, the PCR conditions used were as follows: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 54.5 °C for 15 sec, extension 72 °C for 30 sec, and final extension at 72 °C for 5 min.

The PCR products were run in a 1.5% agarose gel for 30 min at 100 V and amplicons were viewed in a UV transilluminator (BIO-RAD Gel Doc EZ Imager). PCR products of representative isolates were then sent to Macrogen, Korea for gene sequencing. To confirm the DNA sequences of the desired *bla* genes, sequences were aligned with the known sequences in the PUBMED nucleotide database using BLAST.

Table 3: Antimicrobial Susceptibility Testing (AST), PCDDT, and the summary of the different β-lactamase genes detected from the 55 ESBL-producing clinical isolates from Ospital ng Maynila Memorial Medical Center.

Sample code	AST	PCDDT	*bla*$_{CTX-M}$	*bla*$_{OXA}$	*bla*$_{SHV}$	*bla*$_{TEM}$
OSMAp1sputum_*K. pneumoniae*	R	+	+	+	-	-
OSMAp2ETA_*K. pneumoniae*	R	+	+	+	-	-
OSMAp3ETA_*K. pneumoniae*	R	-	+	+	+	+
OSMAp4ETA _*K. pneumoniae*	R	-	+	+	-	-
OMSAp5sputum_*K. pneumoniae*	R	-	+	+	-	-
OSMAp6urine_*K. pneumoniae*	R	+	+	+	-	-
OSMAp7corneal_*E. aerogenes*	R	-	+	+	-	-
OSMAp8sputum_*K. pneumoniae*	R	+	+	+	-	-
OSMAp9sputum_*K. pneumoniae*	R	-	+	+	-	-
OSMAp10sputum_*K. pneumoniae*	R	-	+	+	-	-
OSMAp11ETA _*K. pneumoniae*	R	+	+	+	+	-
OSMA p12urine_*K. pneumoniae*	R	-	+	+	+	-
OSMAp13ETA _*K. pneumoniae*	R	+	+	+	+	-
OSMAp14sputum_*K. pneumoniae*	R	-	+	+	+	-
OSMAp15ETA _*K. pneumoniae*	R	+	+	+	+	-
OSMAp16ETA_*K. pneumoniae*	R	-	+	+	+	-
OSMAp17sputum_*K. pneumoniae*	R	-	+	+	+	+
OSMAp18wound_*K. pneumoniae*	R	-	+	+	+	+
OSMAp19wound_*K. pneumoniae*	R	-	+	-	-	+
OSMAp20sputum_*K. pneumoniae*	R	+	+	+	+	+
OSMAp21corneal_*K. rhinoscleromatis*	R	+	+	+	+	-
OSMAp22urine_*Enterobacter spp.*	R	-	+	+	+	+
OSMAp23urine_*K. rhinoscleromatis*	R	+	+	+	+	+
OSMAp24sputum_*K. pneumoniae*	R	-	+	+	+	+
OSMAp25wound_*Enterobacter spp.*	R	+	+	+	+	+
OSMAp26wound_*Enterobacter spp.*	R	-	+	+	+	+
OSMAp27ETA_*K. rhinoscleromatis*	R	+	+	+	+	+
OSMAp28ETA_*K. pneumoniae*	R	-	+	+	+	+
OSMAp29ETA_*K. pneumoniae*	R	-	+	+	+	+
OSMAp30breastmilk_*K. cloacae*	R	-	+	+	+	+
OSMAp31breastmilk_*K. ozaenae*	R	+	+	+	-	+
OSMAp32wound_*K. pneumoniae*	R	+	+	+	+	+
OSMAp33ETA_*K. pneumoniae*	R	+	+	+	+	+
OSMAp34urine_*E. coli*	R	-	+	+	+	+
OSMAp35sputum_*Klebsiella spp.*	R	+	+	+	+	+
OSMAp36urine_*Enterobacter spp.*	R	+	+	+	+	+
OSMAp37urine_*E. coli*	R	-	+	+	+	+
OSMAp38wound_*E. cloacae*	R	+	+	+	+	+
OSMAp39urine_*Enterobacter spp.*	R	-	+	+	+	+
OSMAp40ETA_*Klebsiella spp.*	R	+	+	+	+	+
OSMAp41sputum_*Enterobacter spp.*	R	-	+	+	+	+
OSMAp42sputum_*E. hafnia*	R	-	+	+	+	+
OSMAp43abscess_*K. pneumoniae*	R	-	+	+	+	+
OSMAp44ETA_*K. pneumoniae*	R	+	+	+	+	+
OSMAp45ETA_*E. aerogenes*	R	-	+	+	+	+
OSMAp46wound_*E. aerogenes*	R	-	+	+	+	+
OSMAp47wound_*Klebsiella spp.*	R	-	+	+	+	+
OSMAp48ETA_*Klebsiella spp.*	R	-	+	+	+	+
OSMAp49sputum_*K. pneumoniae*	R	+	+	+	+	+
OSMAp50wound_*K. pneumoniae*	R	+	+	+	+	-
OSMAp51sputum_*K. rhinoscleromatis*	R	-	+	-	+	+
OSMAp52sputum_*Klebsiella spp.*	R	-	+	+	+	+
OSMAp53sputum_*Klebsiella spp.*	R	-	+	+	+	+
OSMAp54ETA_*Enterobacter spp.*	R	-	+	+	+	+
OSMAp55sputum_*Enterobacter spp.*	R	-	+	+	+	+

Resistant (R) isolates should have been resistant to at least one of the antibiotics used in the antibiotic susceptibility test based on CLSI guidelines. PCDDT (+) are strains that exhibits an increase in the zone diameter of ≥ 5 mm in at least one or both of the third generation cephalosporins used and the disks containing these antibiotics plus clavulanic acid. Gene typing (+) presence of the gene of interest, (–) absence of gene of interest.

RESULTS

Antimicrobial Susceptibility Testing and Identification of isolates

A total of 55 out of 100 clinical isolates from infected patients of Ospital ng Maynila Memorial Medical Center, Philippines were found to be resistant to more than one of the following CLSI recommended beta lactam antibiotics; ampicillin/sulbactam (SAM), piperacillin/tazobactam (TZP), ceftazidime (CAZ), cefotaxime (CTX), ceftriaxone (CRO), and imipenem (IPM) thus, were considered as ESBL-producers. Among these bacterial isolates, 17 came from the sputum, 16 from endotracheal aspartate (ETA), 8 from urine samples, 2 were collected from corneal specimen, 9 from wound discharge, 2 from breast milk, and 1 from abscess (Table 2). These isolates were subjected to standard morphological and biochemical tests

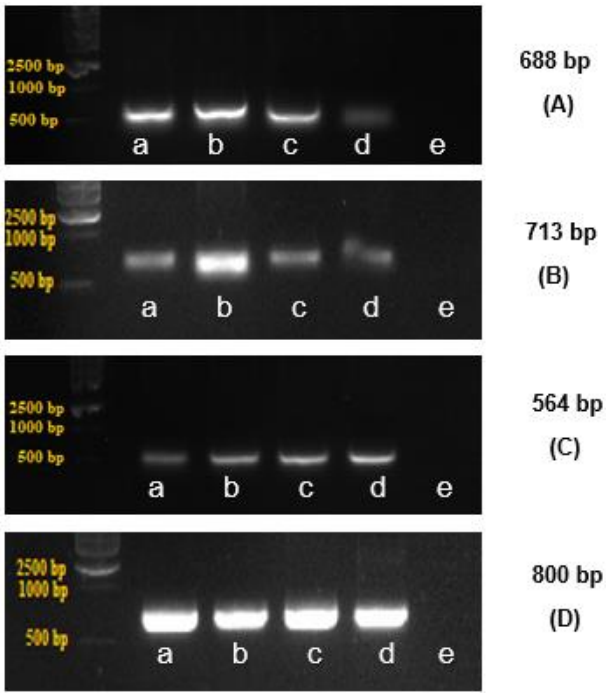

Figure 2: Amplification of the different *bla* genes by Polymerase Chain Reaction (PCR) (A) the 688 bp *bla*$_{CTX-M}$ gene fragment from representative isolates: (a) positive control, (b) OSMAp1sputum_*K. pneumoniae*, (c) OSMAp2ETA_*K. pneumoniae*, (d) OSMAp3ETA_*K. pneumoniae* (e) negative control. (B) the 713 bp *bla*$_{SHV}$ gene fragment from representative isolates: (a) positive control, (b) OSMAp11ETA _*K. pneumoniae*, (c) OSMA p12urine_*K. pneumoniae*, (d) OSMAp13ETA _*K. pneumoniae*, (e) negative control. (C) the 564 bp *bla*$_{OXA}$ gene fragment from representative isolates: (a) positive control, (b) OSMAp4ETA_*K. pneumoniae*, (c) OMSAp5sputum_*K. pneumoniae*, (d) OSMAp6urine_*K. pneumoniae*, (e) negative control. (D) the 800 bp *bla*$_{TEM}$ gene fragment from representative isolates: (a) positive control, (b) OSMAp17sputum_*K. pneumoniae*, (c) OSMAp18wound_*K. pneumoniae*, (d) OSMAp19wound_*K. pneumoniae*, (e) negative control.

for partial characterization. The identities of these isolates were then confirmed by amplification and sequencing of the *16S rRNA* gene. Results showed that the isolates all belonged to the family Enterobacteriaceae with six identified as *Klebsiella spp.*, one *Klebsiella cloacae*, one *Klebsiella ozaenae*, 28 *Klebsiella pneumoniae*, four *Klebsiella rhinoscleromatis*, eight *Enterobacter spp.*, one *Enterobacter hafnia*, one *Enterobacter cloacae*, three *Enterobacter aerogenes*, and two *Escherichia coli* (Table 2).

Confirmation of ESBL Production

Out of the 55 isolates that were initially screened to be resistant to different β-lactam antibiotics, only 22 (40%) were confirmed to be ESBL-producers by PCDDT based on an increase in at least 5 mm in the diameter of the zone of inhibition produced between the disk with the 3rd generation cephalosporin (ceftaxidime or cef$_{OXA}$mine) and the disk containing the antibiotic plus the β-lactamase inhibitor (clavulanic acid) (Table 3). This results showed that majority of the isolates that were initially determined to be ESBL producers by antimicrobial susceptibility testing did not exhibited a positive result for ESBL production based on PCDDT indicating that the ESBL enzymes were not detected by this test. A representative isolate that exhibits positive for ESBL production and a non-ESBL producer are shown in Fig. 1. ESBL-positive for PCDDT was considered if there was increase in at least 5 mm in the diameter of the zone of inhibition produced between the disk with the 3rd generation cephalosporin (ceftaxidime or cef$_{OXA}$mine) and the disk containing the antibiotic plus the β-lactamase inhibitor (clavulanic acid). From of the ESBL-positive isolates, 13 out of 22 (59.1%) were *K. pneumoniae*, three (13.6%) were *K. rhinoscleromatis*, two (9.1%) were *Klebsiella spp*., two (9.1%) were *Enterobacter spp.*, one (4.5%) was *K. ozaenae*, and one (4.5%) *E. cloacae*. Among the different ESBL-producing clinical isolates, the highest number of ESBL-positive isolates was found to be from endotracheal aspirate with eight out of 22 (36.4%), followed by sputum with five (22.7%), wound discharge with four (18.2%), urine with three (13.6%), corneal specimen with one 1 (4.5%), and from breastmilk with one (4.5%). These results therefore confirms that Enterobacteriaceae ESBL-producers were observed in different clinical samples from patients of Ospital ng Maynila Memorial Medical Center, Philippines.

Detection of β-lactamase Genes

The type of β-lactamase genes carried by all the 55 initial ESBL-positive isolates was detected through the amplification of the *bla*$_{CTX-M}$, *bla*$_{OXA}$, *bla*$_{SHV}$, and *bla*$_{TEM}$ genes by Polymerase Chain Reaction (PCR). These genes were selected as they were previously reported to be the most prevalent *bla* genes among Enterobacteriaceae (Barguigua et al. 2011). Results of this study showed that all the different clinical isolates tested possessed the *bla*$_{CTX-M}$ gene suggesting that this might be the most prevalent *bla* gene present among the Enterobacteriaceae strains of Ospital ng Maynila Memorial Medical Center (Table 3; Fig 2A). This was followed by the *bla*$_{OXA}$ gene, which was present in 53 out of 55 (96.4%) bacterial strains (Fig. 2C). Moreover, 44 (80%) of the strains harboured the *bla*$_{SHV}$ gene while 38 (69.1%) possessed the *bla*$_{TEM}$ gene (Fig. 2B; Fig. 2D). The images shown in Fig. 2 are typical examples of positive PCR products along with the control. Results of genotyping therefore confirmed that the different ESBL-positive clinical strains possessed different types of *bla* genes. Moreover, alignment of DNA sequences of the different *bla* genes from representative isolates showed almost 100% identity to deposited *E. coli bla* gene sequences in the nucleotide database (Table 4).

Moreover, results of this study also showed the presence of multiple β-lactamase genes within the same strain of bacteria. Alarmingly, 35 out of the 55 (63.6%) clinical strains possessed all of the four *bla* genes tested. Among the Enterobacteriaceae that possessed all the *bla* genes, *Klebsiella pneumoniae* showed the highest prevalence with 12 out of 35 (34.3%) strains followed by *Enterobacter* spp. and *Klebsiella spp.* with 8 (22.9%) and 6 (17.1%), respectively. Furthermore, from all the 55 clinical isolates 10 (18.2%) carried at least three of the β-lactamase genes with 8 showing the (*bla*$_{CTX-M}$, *bla*$_{OXA}$, *bla*$_{SHV}$) gene combination; one with (*bla*$_{CTX-M}$, *bla*$_{OXA}$, and *bla*$_{TEM}$) and another one with (*bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{TEM}$) gene combinations (Table 5). On the other hand, another 10 (18.2%) of the bacterial strains harboured at least two genes with 9 possessing the (*bla*$_{CTX-M}$; *bla*$_{OXA}$) genes while 1 with (*bla*$_{CTX-M}$; *bla*$_{TEM}$) genes. In this study, *K. pneumoniae* was also observed to have the highest number of clinical strains to possessed three or two of the β-lactamase genes.

However, it was interestingly to note that *bla* genes were still detected even for some isolates that were negative for the PCDDT (Table 3). Thirty three out of the 55 (60%) bacterial strains that were negative for ESBL-production still possessed the *bla* genes. In fact, 22 out of these 33 strains (66.7%) even harboured all the four genes tested in this study (Table 3).

Table 4: BLAST results of percent identity of DNA sequences of the different *bla* genes from selected isolates aligned with known sequences from PUBMED nucleotide database.

Sample code	% Identity	Homologue	Accession
OSMAp1sputum_*K. pneumoniae* bla_{CTX-M}	99.5	*Escherichia coli* strain EPCM-1 $_{CTX-M}$	MG774932.1
OSMAp4ETA _*K. pneumoniae* bla_{OXA}	100	*Escherichia coli* pQGU16 bla_{OXA}	NG 062252.1
OSMAp11ETA _*K. pneumoniae* bla_{SHV}	100	*Escherichia coli* SEC09 bla_{SHV}	NG 05004.1
OSMAp17sputum_*K. pneumoniae* bla_{TEM}	100	*Escherichia coli* strain CR4 $_{TEM-1}$	MK4D5592.1

Table 5: Different β-lactamase gene combinations among Enterobacteriaceae **strains isolated from clinical samples in Ospital ng Maynila Memorial Medical Center, Philippines.**

Bacterial strains	bla_{CTX-M} bla_{OXA}	bla_{CTX-M} bla_{TEM}	bla_{CTX-M} bla_{OXA} bla_{SHV}	bla_{CTX-M} bla_{OXA} bla_{TEM}	bla_{CTX-M} bla_{SHV} bla_{TEM}	bla_{CTX-M} bla_{OXA} bla_{SHV} bla_{TEM}	Total
Klebsiella spp.	-	-	-	-	-	6	(6)
K. cloacae	-	-	-	-	-	1	(1)
K. ozaenae	-	-	-	1	-	-	(1)
K. pneumoniae	8	1	7	-	-	12	(28)
K. rhinoscleromatis	-	-	1	-	1	2	(4)
Enterobacter spp.	-	-	-	-	-	8	(8)
E. hafnia	-	-	-	-	-	1	(1)
E. cloacae	-	-	-	-	-	1	(1)
E. aerogenes	1	-	-	-	-	2	(3)
Escherichia coli	-	-	-	-	-	2	(2)

DISCUSSION

The increasing and rapid development antibiotic resistance among different bacterial strains have continued to pose danger to public health worldwide. The constant exposure of Gram-negative bacteria to a number of β-lactam antibiotics have contributed to the continuous production and mutation of β-lactamases which results to the expanded resistance to β-lactam antibiotics becoming extended-spectrum β-lactamases (ESBLs) (Pitout & Laupland 2008). Large proportion of serious and life threatening infections are now related to ESBL-producing bacteria that are resistant to multiple antibiotics (Partridge 2015). These ESBLs have now spread across the world and have been found in major populated areas (Medeiros 1997). ESBL-producing Enterobacteriaceae were first reported in the middle of 1980s and since then, a steady increase of these strains have been reported worldwide (Bradford 2001). In the Philippines, the prevalence of ESBL-producing Enterobacteriaceae have continuously increased probably due to different bacterial mechanisms of gene horizontal transfer, conjugation, and transposons activity that may spread antibiotic resistance genes. In this study, out of 100 clinical isolates obtained from infected patients of the Ospital ng Maynila Memorial Medical Center, Philippines a total of 55 were found to be resistant to more than one of the β-lactam antibiotics tested thus, were considered as ESBL-producers. These isolates were identified as *Enterobacter aerogenes*, *Enterobacter hafnia*, *Enterobacter spp.*, *Enterobacter cloacae*, *Klebsiella spp.*, *Klebsiella pneumoniae*, *Klebsiella rhinoscleromatis*, *Klebsiella cloacae*, *Klebsiella ozaenae*, and *Escherichia coli*. Majority of the ESBL positive bacterial strains belonged to the genus *Klebsiella* which is consistent with previous reports of being the most commonly reported ESBL-producer. This can be alarming because *Klebsiella* is a major cause of nosocomial diseases and can rapidly spread in hospitals often leading to significant proportion of hospital-acquired infections. Moreover, the colonization of multiple drug resistant *Klebsiella* may pose greater threats to patients as it may entail a more difficult therapeutic intervention strategies.

Of the 55 Enterobacteriaceae clinical strains that were initially determined to be ESBL producers, only 22 (40%) were phenotypically confirmed to be ESBL-positive using PCDDT. Previous studies using PCDDT also showed that ESBL-producers may exhibit false susceptibility to third generation cephalosporins, such as ceftazidime and cefotaxime because these enzymes have wide spectrum of substrate specificity (Mangaiyarkarasi et al. 2013). This might be the reason of the low rate of confirmed ESBL-producers found in this study. Phenotypic confirmatory tests was also shown to exhibit false positive or false negative results based on the study of Khodare et al. (2017).

The vast majority of ESBLs include the CTX-M, OXA, SHV, and TEM types coded by the bla_{CTX-M}, bla_{OXA}, bla_{SHV}, and bla_{TEM} genes, respectively. However, several resistance genes on the same plasmid may enable bacterial cell to acquire multi-resistance in a single step (Paterson and Bonomo 2005). Chromosomal gene mutations may also contribute to the development of antibiotic resistance as sharing of genetic material could be possible through mobile resistance genes (Bradford 2001). Selection of mutations may result in increased resistance to extended spectrum of antibiotics caused by antibiotic pressures (Partridge 2015). Detection and genotyping of the β-lactamase genes is therefore very important to determine the presence and the types of *bla* genes that is predominant in the bacterial cell. In this present study however, *bla* genes were still detected even for those strains negative for PCDDT. It might also be plausible that even if the β-lactamase genes were present but these were not actively expressed by the organism thus, the ESBL enzymes were not detected by PCDDT causing the false negative results. However, future experimental work are needed to further elucidate the possible reasons of the false negative results in PCDDT observed in this study. The use of molecular methods therefore, can be considered as the gold standard as it provides a fast and more accurate method of confirming ESBL-production among bacterial isolates.

In this study, genotyping of the *bla*$_{CTX-M}$ gene showed that it was found in all of the Enterobacteriaceae strains examined thus, can be considered as the most predominant type of β-lactamase gene among the different clinical isolates from the Ospital ng Maynila Memorial Medical Center. This is not surprising because CTX-M ESBLs have begun to emerge in many countries during the past decade and is now considered the most frequent ESBL-type worldwide (Paterson and Bonomo 2005). The CTX-M types of ESBLs are not only predominant in the United States, but also in European and in Asian countries such as China, South Korea and Japan (Zhang et al. 2014). In fact, the CTX-M series enzymes have also been reported to be the most dominant ESBL-type within the Southeast Asian region and has appeared significantly in Vietnam, Thailand and particularly in India (Mendes et al. 2013; Hawkey 2008). In the Philippines, a study conducted by Tian et al. (2010) reported that 37 out of 39 (94.9%) ESBL-producing clinical isolates from the Philippine General Hospital possessed the *bla*$_{CTX-M}$ gene thus, considered as the most predominant extended-spectrum β-lactamases among Enterobacteriaceae isolates similar to this study. Moreover, Lucena et al. (2012) also reported that around 60% of ESBL-producing Enterobacteriaceae at a private tertiary hospital in Southern Philippines produced the $_{CTX-M}$ type ESBLs. The *bla*$_{CTX-M}$ type was also predominantly observed from phenotypically identified ESBL-producing *E. coli* isolates from the Philippines (Cruz and Hedreyda 2017).

On the other hand, among all the clinical isolates genotyped, 53 out of 55 (96.4%) bacterial isolates possessed the *bla*$_{OXA}$ gene. The $_{OXA}$-type is another growing family of ESBLs that confers resistance to ampicillin and cephalothin and are characterized by high hydrolytic activity to $_{OXA}$cillin and cl$_{OXA}$cillin but are poorly inhibited by clavulanic acid (Walther-Rasmussen and Høiby 2006). In the recent study of Cornista et al. (2019), the *bla*$_{OXA-1}$ was reported to be the most predominant *bla* gene from phenotypically confirmed ESBL-positive *K. pneumoniae* isolated from four provincial hospitals in Luzon, Philippines. In this present study, the *bla*$_{OXA}$ was also found in majority of the *K. pneumoniae* clinical isolates from the Ospital ng Maynila Memorial Medical Center similar to our previous report. However, the type of $_{OXA}$ variants was also not determined in this study because DNA sequencing was only used to confirm the presence of this gene.

Furtermore, the *bla*$_{SHV}$ gene was present in 44 out of 55 (80%) ESBL positive clinical isolates. SHV together with TEM β-lactamases, were the first plasmid-mediated enzymes in which most ESBLs were derived (Bush & Jacoby 2010). Majority of SHV-type derivatives have been reported as extended-spectrum β-lactamases (Ojdana et al. 2014). The majority of SHV variants possess an ESBL phenotype characterized by the single amino acid substitution of a serine to glycine at position 238, which is critical for the efficient hydrolysis of ceftazidime. Meanwhile, the substitution of lysine for glutamate at position 240 is critical for the efficient hydrolysis of cefotaxime (Veras et al. 2011). The occurrence of SHV-12 among the ESBL-producing Enterobacteriaceae from clinical isolates at the Philippine General Hospital (PGH) was first reported by Cabrera and Rodriguez (2009). In their study, they concluded that *bla*$_{SHV-12}$ was the most dominant ESBL among the Enterobacteriaceae isolates. The SHV-1 β-lactamase was also reported to be commonly found in *K. pneumoniae* and is responsible for up to 20% of plasmid-mediated antibiotic resistance in this species (Bradford 2001). In this present study however, 19 out of 28 (67.9%) *Klebsiella pneumoniae* also possessed the *bla*$_{SHV}$ indicating that it is common among this strains. This gene was also found in *K. rhinoscleromatis, K. cloacae* and *Klebsiella spp.* clinical isolates from the Ospital ng Maynila Memorial Medical Center.

Lastly, among the ESBL genes tested for this study, the *bla*$_{TEM}$ gene was the least predominant gene present only in 38 out of 55 (69.1%) isolates. The $_{TEM}$-1 enzyme has been reported to be the most commonly encountered β-lactamase among Gram-negative bacteria mostly often found in *K. pneumoniae* and *E. coli.* In this study, the *bla*$_{TEM}$ was present in 13 out of 28 (46.4%) *K. pneumoniae* and in both the *E. coli* clinical isolates. In the study of Cruz and Hedreyda (2017), it was shown that plasmid-encoded *bla*$_{TEM}$ was most prevalent in ESBL-producing *E. coli* isolates in the Philippines which was comparable to *bla*$_{CTX-M}$. In another study of Bajpai et al. (2017), the *bla*$_{TEM}$ gene was also found to be the most predominant gene detected among 38 out of 78 (48.7%) urine isolates. $_{TEM}$-52 was also found to be one of the predominant gene in ESBL-producing *E. coli* and *K. pneumoniae* isolates in South Korea (Pai 1998). However, in most countries in Asia, $_{TEM}$ derivatives still continues to be in scarce, especially in *K. pneumoniae* isolates (Hawkey 2008).

This present study also reported a high prevalence of ESBL-producing Enterobacteriacea strains harboring more than one type of β-lactamase genes which may pose a more serious threat to patients. In fact, 63.6% of all the clinical strains from the Ospital ng Maynila tested possessed all of the four *bla* genes while 18% harboured at least three *bla* gene combinations. Majority of those strains with multiple *bla* genes was *K. pneumoniae* which is a major cause of nosocomial diseases in hospitals. This is not surprising because β-lactamase genes have commonly been reported in *K. pneumonia* strains. The presence of these several antibiotic resistance genes among clinical strains in the Ospital ng Maynila Memorial Medical Center may indicate higher resistance to β-lactam antibiotics thus, might be difficult to manage within the hospital setting.

Studies have also shown that the concomitant presence and hyperproduction of the different types of β-lactamases within the same strain may lead to the development of resistance to β-lactamase inhibitors such as clavulanic acid (Sugumar et al. 2014). Moreover, the association of CTX-M with OXA type of β-lactamases will make isolates resistant to β-lactam-β-lactamase inhibitor combination. In this study, both the *bla*$_{CTX-M}$ and the *bla*$_{OXA}$ genes were found to be very common combination and was present in almost all except for two Enterobacteriaceae strains which might be the reason for the false negative results observed in PCDDT. Here, most of the isolates that were found negative for PCDDT still possessed different types of β-lactamase genes as determined by PCR. This study therefore strengthen the need for molecular screenings for ESBL-positive strains to be applied in routine diagnostics in the Philippines to provide fast and more accurate results. It is also imperative that close monitoring and antimicrobial stewardship must be imposed to further control the spread of the ESBL-producing bacteria within or outside hospital settings as its prevalence has been observed to continuously increase.

CONCLUSION AND RECOMMENDATIONS

From a total of 100 different clinical isolates of the Ospital ng Maynila Memorial Medical Center, Philippines, 55 Enterobacteriaceae strains were found to be resistant to more than one β-lactam antibiotics thus were considered ESBL-suspects. Confirmation of ESBL-production by PCDDT showed that only 22 (40%) out of the 55 strains were ESBL-positive. However, genotyping of isolates based on *bla* genes showed that all the 55 strains possessed β-lactamase genes even those isolates negative for PCDDT. These results suggest that the *bla* genes might be present in all strains but may not be actively expressed thus, the enzyme might not been detected by

PCDDT. Majority of the bacterial strains contained multiple β-lactamase genes with (63.6%) harbouring all the four *bla* genes tested. Among all the Enterobacteriaceae strains, *Klebsiella pneumonia* was found to possessed the highest number of multiple *bla* genes which may pose a greater threat as it is a major cause of nosocomial diseases. This result was not surprising because previous reports have shown that ESBL production is very common in *K. pneumoniae* species. Interestingly, *bla*$_{CTX-M}$ was reported to be present in all of the isolates suggesting that it might be the most dominant gene among the Enterobacteriaceae. This gene was also previously reported to be prevalent in bacterial samples isolated from different hospitals in the Philippines. DNA sequencing of complete *bla* genes from the different isolates is recommended to determine possible variants of these genes. The presence multiple combinations of different types of β-lactamase genes found in bacterial strains in this study therefore reveals the importance of rapid and accurate detection of ESBL-production in healthcare settings.

REFERENCES

Ambler RP, Coulson AF, Frere JM, Ghuysen JM, Joris B, Forsman M, Levesque RC, Tiraby G, Waley SG. A standard numbering scheme for the class A β-lactamase. Biochem J 1991; 276: 269–270.

Barguigua A, El Otmani F, Talmi M, Bourjilat F, Haouzane F, Zerouali K, Timinouni M. Characterization of extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from the community in Morocco. J. Med. Microbiol. 2011; 60: 1344–1352.

Bajpai T, Pandey M, Varma M, Bhatambare GS. Prevalence of TEM, SHV, and CTX-M Beta-Lactamase genes in the urinary isolates of a tertiary care hospital. Avicenna J. Med. 2017; 7(1): 12-16.

Bergey DH, Holt JG, Bergey's Manual of Determinative Bacteriology 9th ed. 1993; Baltimore: Williams & Wilkains.

Bomasang ES, Mendoza MT. Prevalence and risk factors associated with extended spectrum β-lactamase (ESBL) production among selected Enterobacteriaceae isolates at the Philippine General Hospital. Philipp. J. Microbiol. Infect. Dis. 2003; 32(4): 151–158.

Bradford P. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. 2001; 14(4): 933–951.

Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for β-lactamase and its correlation with molecular structure. Antimicrob. Agents Chemother 1995; 39: 1211–1233.

Bush K, Jacoby GA. Updated functional classification of β-lactamases. Antimicrob Agents Chemother 2010; 54(3): 969–976.

Cabrera EC, Rodriguez RDM. First report on the occurrence of SHV-12 extended-spectrum β-lactamase-producing Enterobacteriaceae in the Philippines. J Microbiol Immunol Infect 2009; 42(1): 74–85.

[CLSI] Clinical Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing, 9th informational supplement [M100-S9]. 1999; Wayne, PA: CLSI.

[CLSI] Clinical and Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition [CLSI Document M07-A9]. 2012; Wayne, PA: CLSI. p. 12–13.

[CLSI] Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; twenty-second informational supplement [CLSI document M100-S23]. 2013; Wayne, PA: CLSI. p. 50–51.

Cornista JC, Cuña AM, Sanchez HJ, Balolong MP. Detection of Extended-spectrum β-Lactamase-producing *Klebsiella pneumoniae* Isolated from Four Provincial Hospitals in Luzon and Genotyping of β-Lactamase (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla* $_{SHV}$, and *bla*$_{OXA-1}$) Genes. Philipp. J. Sci. 2019; 148(2):277-287.

Cruz MC, Bacani CS, Mendoza AB, Hedreyda CT. Evaluation of extended-spectrum β-lactamase production in *Escherichia coli* clinical isolates from three hospitals in Luzon, Philippines. Philipp. Sci. Lett. 2014; 7(2): 438–443.

Cruz MC, Hedreyda CT. Detection of plasmid-borne β-lactamase gene in extended-spectrum β-lactamase (ESBL) and non-ESBL-producing *Escherichia coli* clinical isolates. Philipp. J. Sci. 2017; 146(2): 167–175.

Dallenne C, Da Costa A, Decré D, Favier C, Arlet G. Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae. J. Antimicrob. Chemother 2010; 65: 490-495.

Garcia MDM, Norre ILP, Gicana AMN, Tariman RR, Rigby KB. Extended spectrum β-lactamases among Enterobacteriaceae isolated from clinical specimens of a tertiary hospital in 2014. J. Adv. Med. Pharm. Sci. 2016; 8(2): 1–8.

Hawkey PM. Prevalence and clonality of extended-spectrum β-lactamases in Asia. Clin Microbiol Infect 2008; 14(1): 159–165.

Hawser S, Bouchillon S, Hoban D, Badal R, Hsueh P, Paterson D. Emergence of high levels of extended-spectrum-β-lactamase-producing Gram-negative bacilli in the Asia-Pacific region: Data from the Study for Monitoring Antimicrobial Resistance Trends (SMART) program, 2007. Antimicrob Agents Chemother 2009; 53(8): 3280–3284.

Khodare A, Mutha A, Purohit M. Prevalence of extended-spectrum ß-lactamases producing *Escherichia coli* in urinary specimens and their phenotypic detection by modified three-dimensional enzyme extract test: Comparison with the Phenotypic confirmatory disk diffusion test. Indian J Microbiol Res 2017; 4(3): 244-247.

Lota M, Latorre A. A retrospective study on extended spectrum β-lactamase bacteria in the Philippines from 1999–2013. Acta Med Philipp 48(1): 28–35.

Lucena M, Metillo E, Oclarit J. 2012. Prevalence of CTX-M extended spectrum ß-lactamase-producing Enterobacteriaceae at a private tertiary hospital in southern Philippines. Philipp. J. Sci. 2013; 141(1): 117–121.

Mangaiyarkarasi T, Dhandapang SP, Latha R, Baskaran K, Hemalatha G, Vijaya R, Ivvala AS. Comparison of double disc synergy test and phenotypic confirmatory disc diffusion test

for detection of ESBL production and their antimicrobial resistance pattern. Int. J. Appl. Biol. Pharm. Tech. 2013; 4(2): 35–39.

Medeiros A. Evolution and dissemination of β-lactamases accelerated by generations of β-lactam antibiotics. Clin. Infect. Dis. 1997; 24(Suppl. 1), S19–S45. 10.1093/clinids/24.Supplement_1.S19

Melzer M, Petersen I. Mortality following bacteraemic infection caused by Extended-spectrum beta-lactamase (ESBL) producing *E. coli* compared to non-ESBL producing *E. coli.* J. Infect. 2007; 55(3): 254-9.

Mendes RE, Mendoza M, Banga Singh KK, Castanheira M, Bell JM, Turnidge JD, Lin SS, Jones RN. Regional resistance surveillance program results for 12 Asia-Pacific nations (2011). Antimicrob Agents Chemother 2013; 57(11): 5721–5726.

Modi D, Patel D, Patel S, Jain M, Bhatt S, Vegad MM. Comparison of various methods for the detection of extended-spectrum β-lactamase from *Klebseilla pneumoniae* isolated from neonatal intensive care unit (Ahmedabad). Nat J Med Res 2012; 2(3): 348–353.

Ojdana D, Sacha P, Wieczorek P. The Occurrence of *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, and *bla*$_{\text{TEM}}$ genes in Extended-Spectrum β-Lactamase-Positive Strains of *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* in Poland. Int. J. Antibiot., 2014: 1-7.

Partridge, S. Resistance mechanisms in Enterobacteriaceae. J Pathol 2015; 47(3): 276–284

Paterson D, Bonomo R. Extended-spectrum β-lactamases: A clinical update. Clin. Microbiol. Rev. 2005; 18(4): 657–686.

Pitout JD, Laupland KB. Extended-spectrum beta-lactamase-producing Enterobacteriaceae: An emerging public health concern. The Lan. Infect. Dis. 2008; 8(3): 159–166.

Salandin M, Cao VT, Lambert T, Donay JL, Herrmann JL, Ould-Hocine Z, Verdet C, Delisle F, Philippon A, Arlet G. Diversity of CTX-M beta-lactamases and their promoter regions from Enterobacteriaceae isolated in three Parisian hospitals. FEMS Microbiol. Lett. 2002; 209(2):161-8.

Schmitt J, Jacobs E, Schmidt H. Molecular characterization of Extended-spectrum Beta-lactamases in Enterobacteriaceae from patients of two hospitals in Saxony, Germany J. Med. Microbiol. 2007; 56(2): 241-249.

Shahi SK, Singh VK, Kumar A. Detection of *Escherichia coli* and Associated β-Lactamases Genes from Diabetic Foot Ulcers by Multiplex PCR and Molecular Modeling and Docking of SHV-1, TEM-1, and OXA-1 β-Lactamases with Clindamycin and Piperacillin-Tazobactam. 2013. PLoS ONE 8(7): e68234. https://doi.org/10.1371/journal.pone.0068234

Spanu T, Luzzaro F, Perilli M, Amicosante G, Tiniolo A, Fadda G. Occurrence of extended-spectrum beta-lactamase in members of the family Enterobacteriaceae in Italy: implications for resistance to beta-lactams and other antimicrobial drugs. Antimicrob Agents Chemother. 2002; 46(1):196-202.

Sugumar M, Kumar KM, ManoharanA, Anbarasu A, Ramaiah S. Detection of OXA-a β-lactamase gene of *Klebsiella pneumoniae* from blood stream infections (BSI) by conventional PCR and *in silico* analysis to understand the mechanism of OXA mediated resistance PLoS One 2014; 9(3): 1–8.

Tham J. Extended-spectrum β-lactamase-producing Enterobacteriaceae: Epidemiology, risk factors, and duration of carriage. Sweden: Media-Tryck, Lund University 2012.

Tian G, Garcia J, Adams-Haduch J, Evangelista J, Destura R, Wang H, Doi Y. CTX-M as the predominant extended-spectrum β-lactamases among Enterobacteriaceae in Manila, Philippines. J Antimicrob Chemother 2010; 65(3): 584–586.

Varkey DR, Balaji V, Abraham J. Molecular characterization of extended-spectrum β-lactamase producing strains from blood sample. Int J Pharm Pharm Sci 2014; 6(3): 276–278.

Villanueva FD, Tupasi TE, Abiad HG, Baello BQ, Cardano RC. Extended-spectrum β-lactamse production among *Escherichia coli* and *Klebseilla* spp. at the Makati Medical Center; tentative solutions. Philipp. J. Microbiol. Infect. Dis. 2003; 32(3): 103–108.

Walther-Rasmussen J, Hoiby N. OXA-type carbapenemases. J. Antimicrob. Chemother. 2006; 57(3): 373–383.