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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

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Extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* is one of the most common cause of nosocomial infections. One hundred isolates of *K. pneumoniae* were obtained from four different provincial hospitals in Luzon. The strains following purification and standard bacteriological testing were then initially screened for antimicrobial susceptibility against five 3rd generation cephalosporins and monobactam. Twenty-three isolates (23%) were initially found to be resistant to all or at least three antibiotics tested. To prove ESBL-production, the phenotypic confirmatory disk diffusion test (PCDDT) was conducted confirming 18 out of the 23 (78.3%) isolates to be ESBL producers. The identity of the isolates was confirmed as *K. pneumoniae* by the amplification and sequencing of the 16S *rRNA* gene through polymerase chain reaction (PCR). To determine the type of the β-lactamase genes carried by the ESBL-producing *K. pneumoniae* isolates, the *bla*$_{CTXM}$, *bla*$_{OXA-1}$, *bla*$_{SHV}$, and *bla*$_{TEM}$ were amplified and sequenced. This study showed that *bla*$_{CTX-M}$ and *bla*$_{TEM}$ were detected in 10 out of 18 ESBL-positive isolates (56%), while *bla*$_{SHV}$ was found in 15 out of 18 isolates (83.3%). Interestingly, the *bla*$_{OXA-1}$ gene was detected in all phenotypically confirmed ESBL isolates suggesting that it was the most predominant β-lactamase gene among the samples. Eight of the isolates harbored at least three genes; four of these isolates have *bla*$_{CTX-M}$, *bla*$_{OXA-1}$, and *bla*$_{SHV}$; three of the isolates have *bla*$_{TEM}$, *bla*$_{OXA-1}$, and *bla*$_{SHV}$; and one with *bla*$_{CTX-M}$, *bla*$_{OXA-1}$, and *bla*$_{TEM}$. Lastly, five isolates harbored all the four genes tested – suggesting that these isolates pose a serious threat in the healthcare industry because of its resistance to a wider range of antibiotics. Because of the occurrence of multiple β-lactamase genes in *K. pneumoniae* , there is therefore an urgent need to develop a rapid and accurate method of ESBL genotyping.

Keywords: 16S *rRNA* gene, *bla*$_{CTX-M}$, *bla*$_{OXA-1}$, *bla*$_{SHV}$, *bla*$_{TEM}$, extended-spectrum β-lactamase (ESBL), *Klebsiella pneumoniae*

INTRODUCTION

The extensive and irrational use of antibiotics have led to the emergence of antibiotic resistance in bacteria that has become a growing problem in the fields of microbiology and medicine worldwide (Lota and Latorre 2013). The rapid mutation, evolution, and spread of resistance genes among microbial species have increased the incidence of ESBL-producing bacteria, which has become a public health concern in the Philippines. ESBLs are enzymes that provide resistance against more than one β-lactam

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antibiotics and are produced by some bacteria that target and hydrolyze the β-lactam ring of the antibiotic, making it ineffective. ESBL-producing bacteria are most commonly found in species belonging to family Enterobacteriaceae – in which members commonly cause diseases and infections such as septicaemia, urinary tract infection, pneumonia, wound infections, meningitis, gastroenteritis, and sporadic outbreaks (Tham 2012). Currently, incidents of ESBLs in members of the family Enterobacteriaceae are continuously increasing worldwide.

K. pneumoniae is one of the most common ESBL-producing bacteria belonging to family Enterobacteriaceae. This bacterium commonly causes respiratory tract infections and post-surgery complications in immunocompromised patients (Newire *et al.* 2013). *K. pneumoniae* is also the most common cause of hospital-acquired infections. To treat such bacterial infections, physicians worldwide use advanced β-lactam antibiotics – particularly 3rd generation cephalosporins such as ceftriaxone, ceftazidime, cefotaxime, cefixime, and cefpodoxime. However, the number of outbreaks involving *K. pneumoniae* with ESBL-mediated resistance to cephalosporins has been progressively increasing in many parts of the world. Pharmaceutical institutions are forced to produce advanced classes of drugs to deal with the rapid evolution of resistance genes. Unfortunately, ESBL-containing bacteria are able to continuously mutate and cope with these advancements by constantly developing new types and variants.

Today, incidents of ESBLs are continuously increasing worldwide due to the discovery of numerous types of these enzymes. β-Lactamases are commonly classified according to molecular or functional classification schemes. The Ambler molecular classification divides β-lactamases into four major classes (A–D) based on amino acid similarity. β-Lactamases A, C, and D are considered as serine β-lactamases while class B are metallo-β-lactamases (Ambler *et al.* 1991). The Bush-Jacoby-Medeiros functional classification, on the other hand, groups β-lactamases according to similarities in substrate or inhibitor profile (Bush *et al.* 1995). The 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). The extended-spectrum β-lactamases mainly arise due to mutations in β-lactamases, which are encoded by the *bla* genes. To date, more than 300 different ESBL variants have already been described (Varkey *et al.* 2014). Studies have concluded that the four most prevalent types of β-lactamase genes are the bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{OXA} – and variants derived from these enzymes form new dominant ESBLs (Barguigua *et al.* 2011). The TEM and SHV-type of ESBLs are the most common plasmid-mediated β-lactamases – often found in *E. coli* and *K. pneumoniae* but have also been found in other genera of Enterobacteriaceae (Bradford 2001). However, strains expressing 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). Meanwhile, the OXA-type are another growing family of ESBLs originally discovered in *P. aeruginosa* but was also found in *E. coli* isolates. It confer resistance to ampicillin and cephalothin and are characterized by high hydrolytic activity to oxacillin and cloxacillin, and are poorly inhibited by clavulanic acid (Walther-Rasmussen and Høiby 2006). Molecular classification groups TEM, SHV, and CTX-M all belong to class A enzymes, whereas OXA belongs to class D β-lactamases. In functional classification, however, TEM-1 and SHV-1 are placed in group 2b; CTX-M in group 2be; and OXA-1 in group 2d.

The prevalence of extended-spectrum β-lactamase in Enterobacteriaceae are increasingly reported worldwide (Lota and Latorre 2014). This is probably due to different bacterial mechanisms such as gene horizontal transfer, conjugation, and the transposons activity that quickly spread antibiotic resistance genes to different parts of the world and to other species of bacteria (Bradford 2001). In Europe, 85–100% ESBL occurrence in *E. coli* and *K. pneumoniae* have been reported while in the United States, around 19% cases of infections caused by ESBL-producing Enterobacteriaceae were observed (Lota and Latorre, 2014). In Latin America, the prevalence among ESBL-producing *K. pneumoniae* is at 45.4–51.9 % while in the Asia Pacific region, the reported ESBL prevalence of *K. pneumoniae* is 35.8% (Hawser *et al.* 2009). Lastly, in the Philippines, the Antimicrobial Resistance Surveillance Program (ARSP) for 2016 estimates the prevalence of ESBL-producing *K. pneumoniae* at 40%.

Reports on the genotyping of β-lactamase genes, particularly of *K. pneumoniae*, in the Philippines is also very limited. The only study conducted on *K. pneumoniae* was on the emergence of carbapenem-resistant *K. pneumoniae* carrying the bla_{NDM-7} and bla_{NDM-1} genes, which are metallo-β-lactamases that have recently emerged as a global health threat (Chou *et al.* 2016). Although local studies have detected bla_{SHV-12}, bla_{CTX-M}, and bla_{TEM} genes among ESBL-producing clinical isolates of members of *Enterobacteriaceae* (Cabrera and Rodriguez 2009, Tian *et al.* 2010, Cruz and Hedreyda 2017), no studies have been reported on the occurrence of bla_{OXA-1} gene – which prompted this study to detect not only the most common bla_{TEM}, bla_{SHV}, and bla_{CTX-M} but also the bla_{OXA-1} gene among ESBL-producing *K. pneumoniae*.

Because of the importance of *K. pneumoniae* in the hospital setting and its ability to harbor and transfer plasmid-mediated ESBL genes quickly, results of this

study may provide baseline data on how far resistance genes have already spread in medical institutions – particularly in four provincial hospitals in Luzon. Moreover, the detection of ESBL production is highly significant in the Philippine setting because of the poor monitoring system of antibiotic use in the country. The data obtained from this study can be used as a basis of practitioners in deciding future therapeutic interventions, which can be an answer in coping with fast evolution of ESBL-producing *K. pneumoniae.*

METHODS

Sample Collection

This study was conducted after obtaining approval from the Ethics Committee of Miriam College and the hospitals involved. Samples were collected from different areas (Emergency Room, ICU, pediatric ward, surgical ward, male and female wards) from selected hospitals in Batangas, Bulacan, Nueva Ecija, and Rizal provinces. ESBLs have been reported to be common in members of the family Enterobacteriaceae particularly *E. coli*; however, *K. pneumoniae* was actively selected because this bacteria is one of the most medically important and the most frequently isolated Gram-negative bacteria in hospital infections. ESBLs are also most commonly detected in *K. pneumoniae*, which is an opportunistic pathogen associated with severe infections in hospitalized patients – including immunocompromised hosts with severe underlying diseases.

Bacteria were isolated by either swabbing or exposing *Klebsiella* Selective Agar (KSA) in highly touched surfaces such as door knobs, faucet, sink, stairs, and even in public areas such as comfort rooms. A clinical sample was also obtained from endotracheal tubes of a patient with respiratory disorder. All samples were transported immediately in a thermal cooler to the Microbiology Research Laboratory of Miriam College, Quezon City, Philippines. KSA plates were incubated at 37 °C for 24 h and a total of 100 isolated colonies were randomly selected. These colonies were first purified in nutrient agar (NA) and characterized by standard bacteriological techniques.

An ESBL-producing Enterobacteriaceae collected from a patient at the Philippine General Hospital harboring the bla_{SHV} gene was provided by Dr. Esperanza Cabrera of De La Salle University, Manila, Philippines (Cabrera and Rodriguez 2009). The positive control was confirmed to be ESBL through the double-disk synergy test. The presence of bla_{SHV} was confirmed by polymerase chain reaction, sequencing, and isoelectric focusing (Cabrera and Rodriguez 2009). The non-ESBL-producing *K. pneumoniae* strain used as a negative control was isolated from a soil sample and was obtained from the Miriam College Culture Collection, Quezon City, Philippines.

Antimicrobial Susceptibility Testing

ESBL activity of the different isolates were detected using standard disk diffusion susceptibility test method with cefpodoxime (10 µg), ceftazidime (30 µg), aztreonam (30 µg), cefotaxime (30 µg), and ceftriaxone (30 µg) disks. Briefly, samples were purified on NA plates and two to three distinct colonies were inoculated in Luria Bertani (LB) broth and incubated at 37 °C for 16–18 h. The suspension was then adjusted with saline solution to achieve turbidity of 0.5 McFarland standard (CLSI 2012a). Next, the cultures were swabbed onto Mueller Hinton agar (MHA) plates (HI-Media Laboratories, India) and disks containing different antibiotics were placed on top of the swabbed portion and then incubated overnight at 37 °C in an upright position. The clearing zones were measured and interpreted based on the guidelines set by the Clinical Laboratory Standards Institute (CLSI 2012b). Isolates that exhibited zone of inhibition that measures <17mm for cefpodoxime, <22mm for ceftazidime, <27mm for aztreonam and cefotaxime, and <25mm for ceftriaxone putatively – suggesting ESBL production and were further selected for the next experiment.

PCDDT for ESBL Production

The presence of ESBL was then confirmed by PCDDT as recommended by CLSI (1999). Bacterial isolates that were previously tested positive by the antimicrobial susceptibility test were further subjected to PCDDT on MHA to confirm ESBL production. Overnight culture of the isolates were grown in LB broth and the suspension was then adjusted with saline solution to achieve turbidity of 0.5 McFarland standard. The suspension was swabbed onto the entire surface of two MHA plates. Then, disks containing cefotaxime (30 µg) and cefotaxime-clavulanic acid (30/10 µg) were placed on one MHA plate, while disks containing ceftazidime and ceftazidime-clavulanic acid (30/10 µg) were placed on the other plate. Disks were positioned side by side at a distance of 30 mm from each other and incubated for 18 hours at 37 °C. A zone of inhibition was measured and an increase in the zone diameter of ≥ 5 mm for either antimicrobial agent tested in combination with clavulanic acid over that when tested alone indicates the ESBL-producing capability of the isolate.

Confirmation of Identity of ESBL-producing Isolates

The identity of the representative ESBL-producing isolates per hospital was confirmed by amplification and sequencing of the 16S *rRNA* gene through PCR.

The genomic DNA was extracted by GF-1 Genomic Extraction Kit (Vivantis, Malaysia). The extracted DNA was then used as a template for 16S *rRNA* gene amplification using Quanta SI-96 Thermocycler. The following forward (5' GCA CAA GCG GTG GAG CAT GTGG 3') and reverse (5' GCC CGG GAA CGT ATT CAC CG 3') universal primers for eubacterial 16S rDNA were used (Kawai *et al.* 2002). The total working volume per PCR reaction was 25 µL with the following composition: 12.5 µL KAPA HiFiHot Start Ready Mix, 1 µL 16S rRNA gene forward primer, 1 µL 16S rRNA gene reverse primer, 8.5 µL ultra-pure water, and 2 µL extracted bacterial DNA. The following PCR conditions were used: a) initial denaturation at 95 °C for 5 min; b) 35 cycles of denaturation (30 s at 94 °C), annealing (30 s at 50 °C), and extension (1 min at 72 °C); and c) a final extension at 72 °C for 5 min. The presence of amplified fragments was checked using agarose gel electrophoresis (AGE) using 1.5% agarose gel and run for 30 min at 100 V. Amplicons from representative isolates were sent to Macrogen, Korea for DNA sequencing.

The gene sequences of the representative isolates were then aligned using Basic Local Alignment Sequencing Tool (BLAST), and the % homologies and possible identities were determined.

Detection of β-Lactamase Genes

The type of β-lactamase genes that might be responsible for the ESBL production of the *K. pneumoniae* isolates were determined by amplification of the $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, and $bla_{\text{OXA-1}}$ genes by PCR. The following sets of primers were used as shown in Table 1.

In all of the genes detected, the reaction mixture amounting to 25 µL is as follows: 1 µL each of forward and reverse primers, 2 µL of the extracted genomic DNA of the samples, 8.5 µL sterile ultra-pure H_2O, and 12.5 µL of KAPAHiFi Hot Start Ready Mix. For the amplification of $bla_{\text{CTX-M}}$ and $bla_{\text{OXA-1}}$ genes, the following PCR conditions were used: 94 °C for 4 min (initial denaturation), 30 cycles of denaturation at 94 °C for 1 min, 55 °C for 20 s (annealing), and 72 °C for 30 s (extension) plus 72 °C for 5 min (final extension). On the other hand, for bla_{SHV} gene amplification, the following PCR conditions were used: initial denaturation at 94 °C for 4 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 57.5 °C for 20 s, extension at 72 °C for 30 s, and final extension at 72 °C for 5 min. Lastly, for the detection of bla_{TEM}, the PCR conditions used were as follows: initial denaturation (94 °C for 4 min), 30 cycles of denaturation (94 °C for 1 min), annealing (59 °C for 20 s), extension (72 °C for 25 s), and final extension (72 °C for 5 min).

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

RESULTS

Collection of Isolates and Antimicrobial Susceptibility Testing

A total of 100 isolates of *K. pneumoniae* consisting of 25 isolates from each hospital in Batangas, Bulacan, Nueva Ecija, and Rizal provinces were randomly selected.

The isolates were initially identified based on their characteristic morphology on selective media. The isolates generally appeared as mucoid with purple-magenta color of different shapes and sizes in KSA (Fig. 1A). The selected isolates were then further subjected to standard morphological and biochemical tests.

In this study, isolates that were resistant to at least two of the 3rd generation cephalosporins and monobactam were selected as ESBL producers and were further subjected to confirmation by PCDDT. Out of the 100 isolates, 23 were found to be resistant to all or at least three of the five antibiotics tested. All 23 isolates showed hydrolytic activity against ceftazidime and ceftriaxone while 22/23 (95.7%) were resistant to cefotaxime and aztreonam. Twenty of the 23 isolates (87.0%) had hydrolytic activity against cefpodoxime (Table 2). The resistance rate of the putative *K. pneumoniae*

Table 1. Summary of primers used for the amplification of different ESBL genes.

Gene	Forward Primer (5 →3')	Reverse Primer (5'→3')	Expected Size (bp)	Reference
$bla_{\text{CTX-M}}$	5'-SCS ATG TGC AGY ACC AGT AA-3'	5'-ACC AGA AYV AGC GGB GC-3'	585	Ojdana *et al*. 2014
bla_{TEM}	5'-GCT CAC CCA GAA ACG CTG GT-3'	5'-CCA TCT GGC CCC AGT GCT GC-3'	686	Ojdana *et al*. 2014
bla_{SHV}	5'-CCC GCA GCC GCT TGA GCA AA 3'	5'-CAT GCT CGC CGG CGT ATC CC-3'	733	Ojdana *et al*. 2014
$bla_{\text{OXA-1}}$	5′-CTG TTG TTT GGG TTT CGC AAG-3′	5′-CTT GGC TTT TAT GCT TGA TG-3′	519	Shi *et al*. 2008

*(S) can be replaced by the base G or C; (Y) with C or T; (V) with A or C or G; and (B) with C or G or T.

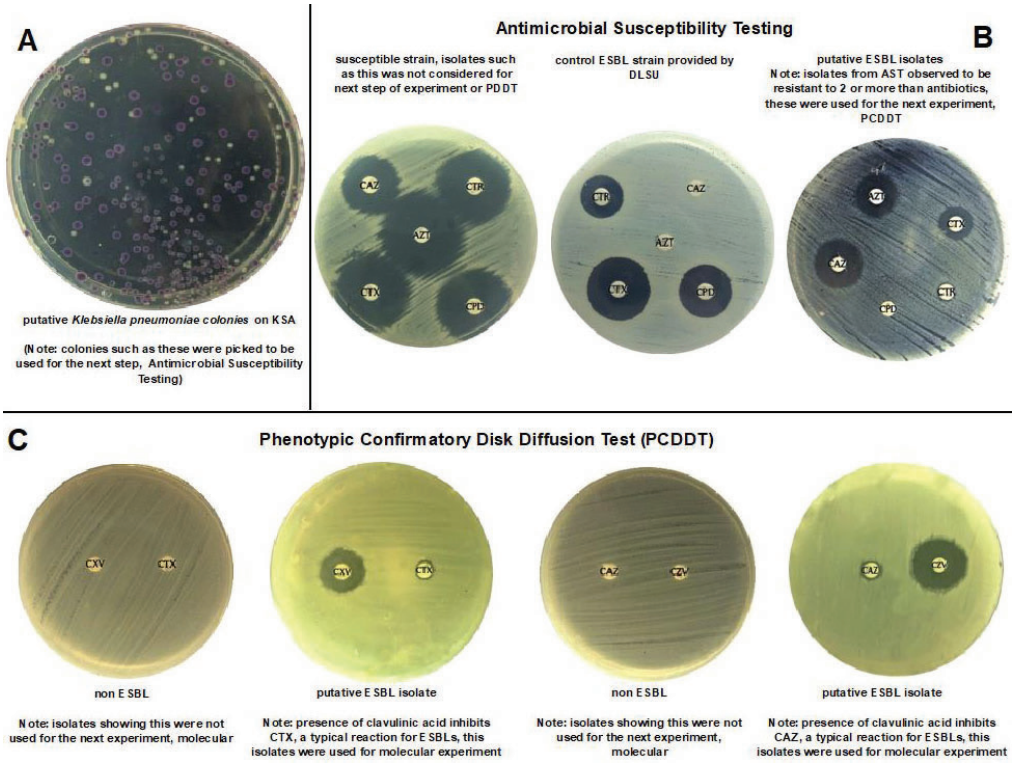

Figure 1. (A) Growth of *K. pneumoniae* isolates in *Klebsiella* Selective Agar (KSA), purple-magenta colonies of different shapes and sizes. (B) Typical observations for antimicrobial susceptibility testing (AST) for typical KP isolates against third generation cephalosporins (ceftazidime, cefotaxime, cefpodoxime, ceftriaxone) and monobactam (aztreonam). (C) Typical observations for PCDDT of isolates that showed putative ESBL characteristics from AST, discs containing cefotaxime (CTX) plus cefotaxime-clavulanic acid (CXV), and ceftazidime (CAZ) plus ceftazidime-clavulanic acid (CZV) were used. Complete isolate profiles based from AST and PCDDT are shown in Tables 2 and 3.

showed the highest against ceftazidime, which was not surprising because this is the most commonly used antibiotic in the initial screening of ESBL cases (Singh *et al.* 2011).

PCDDT for ESBL Production

Of the 23 putative *K. pneumoniae* isolates that were initially screened positive for ESBL production, 18 were confirmed to be ESBL-positive using PCDDT – as shown by 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 cefoxamine) and the disk containing the antibiotic plus the β-lactamase inhibitor (clavulanic acid). Representative results are described in Figures 1B–G. All isolates from Batangas and Nueva Ecija that were initially screened to be β-lactamase-producers by the antimicrobial susceptibility test were all confirmed to be ESBL-positive by PCDDT. However, three out of seven (42.9%) isolates from a hospital in Bulacan and two out of seven (28.6%) from Rizal that were initially screened positive for β-lactamase production tested negative by PCDDT (Table 2). These results showed that although antibiotic susceptibility test is routinely used as a standard method for detecting antibiotic resistance, it may provide a false positive result – making PCDDT a more sensitive and effective method in detecting ESBL production (Sharma *et al.* 2013).

For the distribution of ESBL producers from the four different provincial hospitals in Luzon, Nueva Ecija and Rizal showed the highest rate of ESBL-producers with five out of 25 (20%) – while four out of 25 (16%) were observed from hospital in Batangas and Bulacan (Table 3). These results suggest that ESBL producers are almost equally prevalent in different hospitals located in Northern Luzon, Philippines.

Detection of β-lactamase Genes from *K. pneumoniae*

The identity of the representative ESBL-producing isolates from different hospitals was confirmed to be *K. pneumoniae* by the amplification and sequencing of the 16S *rRNA* gene. The type of *bla* genes carried by the 18 ESBL-positive *K. pneumoniae* were detected by PCR amplification of the $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, and $bla_{\text{OXA-1}}$ genes. In the Philippines, there are also very limited studies on ESBL-

Table 2. Antimicrobial susceptibility test of putative *K. pneumoniae* isolates against selected 3rd generation cephalosphorin and monobactam, PCDDT, and the summary of the different ESBL and β-lactamase genes detected from the 18 ESBL-producing *K. pneumoniae* isolates from four different hospitals in Luzon.

Sample site	Sample code	AZT	CAZ	CPD	CTR	CTX	PCDDT	bla_{CTX-M}	bla_{OXA}	bla_{SHV}	bla_{TEM}
Batangas	BAT_FCRDoorKnob	R	R	R	R	R	+	+	+	+	+
	BAT_PediaWard2	R	R	R	R	R	+	+	+	+	–
	BAT_PediaWard3	R	R	R	R	R	+	+	+	+	+
	BAT_SurgWard3	R	R	R	R	R	+	+	+	+	+
Bulacan	BUL_IsoCR7	R	R	R	R	R	+	–	+	+	+
	BUL_IsoPublicCR	R	R	R	R	R	+	+	+	+	–
	BUL_ICUVent**	R	R	R	R	R	+	+	+	–	+
	BUL_ICU	R	R	R	R	R	–	*	*	*	*
	BUL_FCRDoorKnob	R	R	R	R	R	+	+	+	+	+
	BUL_ERDoorKnob	R	R	R	R	R	–	*	*	*	*
	BUL_IsoWard	R	R	R	R	R	–	*	*	*	*
Nueva Ecija	NE_NewbornWard	R	R	S	R	R	+	–	+	+	–
	NE_OBWardFaucet1	R	R	S	R	S	+	+	+	+	+
	NE_FemaleWard2	S	R	R	R	R	+	–	+	+	+
	NE_MaleSurgWard3	R	R	S	R	R	+	+	+	+	–
	NE_OBWardFaucet2	R	R	R	R	R	+	–	+	+	+
Rizal	RIZ_PediaIsoRoom1	R	R	R	R	R	+	–	+	–	–
	RIZ_PediaC1	R	R	R	R	R	–	*	*	*	*
	RIZ_ER1	R	R	R	R	R	–	*	*	*	*
	RIZ_Lobby1	R	R	R	R	R	+	–	+	–	+
	RIZ_OBCS	R	R	R	R	R	+	+	+	+	–
	RIZ_IsoRoom	R	R	R	R	R	+	–	+	+	–
	RIZ_MaleWard4	R	R	R	R	R	+	–	+	+	–

Resistant (R) samples should have zones of inhibition that are ≤27mm in aztreonam (AZT), ≤22mm in ceftazidime (CAZ), ≤17mm in cefpodoxime (CPD), ≤25 in ceftriaxone (CTR), and ≤27mm in cefotaxime (CTX). Susceptible (S) bacteria, on the other hand, have zone of inhibition wider than the standard. PCDDT: (+) an increase in the zone diameter of ≥ 5 mm for either CAZ or CTX and the disk containing these antibiotics plus clavulanic acid, while (–) is less than 5 mm increase. Gene typing: (+) presence of the gene of interest, (–) absence of gene of interest. (*) Not tested since result was negative in PCDDT. (**) BUL_ICUVent is a clinical isolate from endotracheal tube of a patient with respiratory disorder.

producing *K. pneumoniae* – particularly on genotyping of *bla* genes. Results of this present study showed that 10 out of 18 (56 %) of *K. pneumoniae* isolates carried the bla_{CTX-M} gene (Figure 2A). Results of the prevalence of the bla_{TEM} among the ESBL-producing *K. pneumoniae* was similar to that bla_{CTX-M} at 56% (Figure 2B). As early as 1990s, TEM- and SHV-type of β-lactamases were considered to be the most dominant ESBL often encountered in clinical isolates of *K. pneumoniae*. Moreover, 15 out of 18 (83%) ESBL-positive *K. pneumoniae* isolates possessed the bla_{SHV} gene (Figure 2D). Interestingly, results of this present study showed that all of the ESBL-positive *K. pneumoniae* isolates tested harbored the bla_{OXA-1} gene (Figure 2C). This result suggests that the bla_{OXA-1} was the most predominant β-lactamase gene and might already be widely distributed in the different hospitals in Luzon, Philippines. In contrast with previous local findings, this is the first report on the predominance of the bla_{OXA-1} type β-lactamase in phenotypically confirmed ESBL-producing *K. pneumoniae* isolated from the Philippines.

DISCUSSION

In this study, *K. pneumoniae* were mostly obtained from air and fomites because this organism is an opportunistic pathogen and has emerged as an important cause of many nosocomial infections (Gupta *et al.* 2003). *K. pneumoniae* can be directly passed through highly touched surfaces and can also go into the air and be transmitted from clinical setting into the natural environment (Huijbers *et al.* 2015). Reservoirs for the transmission of *K. pneumoniae* are normally through the hands of patients and hospital

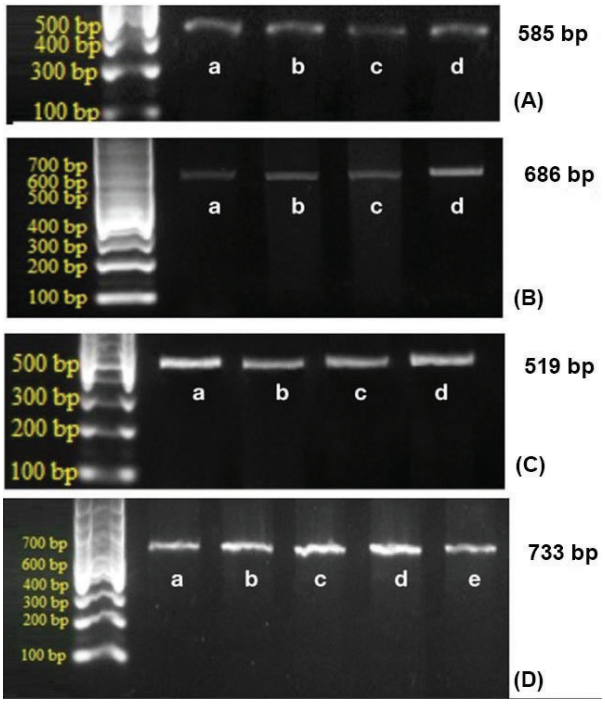

Figure 2. (A) Amplification of the 585 bp bla_{CTX-M} gene fragment from representative isolates: (a) BAT_FCRDoorKnob; (b) BUL_FCRDoorKnob; (c) NE_OBWardFaucet1; (d) RIZ_OB_CS. (B) The 686 bp bla_{TEM} gene fragment from representative isolates: (a) BAT_FCRDoorKnob; (b) BUL_FCRDoorKnob; (c) NE_OBWardFaucet1; (d) RIZ_Lobby2. (C) The 519 bp bla_{OXA-1} gene fragment from representative isolates: (a) BAT_FCRDoorKnob; (b) BUL_FCRDoorKnob; (c) NE_OBWardFaucet1; (d) RIZ_OB_CS. (D) The 733 bp bla_{SHV} gene fragment from representative isolates: (a) BAT_FCRDoorKnob; (b) BUL_FCRDoorKnob; (c) NE_OBWardFaucet1; (d) RIZ_OB_CS; (e) positive control.

personnel. Breathing and sneezing can also release *K. pneumoniae* into the air and can remain airborne for several hours, thus posing a risk as a contagious respiratory pathogen. The poor cleanliness and hygienic practices of some hospital personnel and patients, coupled with poor hospital disinfection techniques, might cause pathogens to spread across different areas in various hospitals in the country. The rapid spread of *K. pneumoniae* in the hospital often leads to significant proportion of hospital-acquired urinary tract infections, pneumonia, septicemias, and soft tissue infections. The high rate of nosocomial *Klebsiella* colonization was reported to be associated with the use of antibiotics, the carriage rate among the hospital personnel, and the type of disinfection used by the hospital (Singh *et al.* 2011).

Of the 23 putative *K. pneumoniae* isolates that were initially screened positive for ESBL production, 18 (78.2%) were confirmed to be ESBL-positive using PCDDT. The use of disc diffusion methods, although used as standard method for detecting ESBL, may sometimes show false susceptibility results because these enzymes exhibit a wide spectrum of substrate specificity (Mangaiyarkarasi *et al.* 2013). Thus, PCDDT is still considered to be the most specific method for the detection of ESBL in *K. pneumoniae* (Modi *et al.* 2012). In a retrospect study on extended-spectrum β-lactamase bacteria, it was shown that in the Philippines there was an increase in the prevalence of ESBL-producing *K. pneumoniae* – ranging 10–42% from 1999 to 2013 (Lota and Latorre 2014). A more recent report of the ARSP of the Department of Health for 2016 showed that the prevalence of ESBL-producing *K. pneumoniae* suspects was around 40% which was relatively higher than the previous year at 38%. Surveillance studies have also shown the presence of ESBL in several countries is increasing with varying frequencies (Lota and Latorre 2014). In the Asia Pacific region, a prevalence rate of 35.8% of ESBL *Klebsiella* spp. was observed, which is almost similar to the data obtained in the Philippines (Hawser *et al.* 2009). Although ESBL rates in the Asia Pacific region has been reported to be relatively high as a whole, several countries such as India (69.4%) and Thailand (45.5%) have strikingly higher rates. Other countries such as Vietnam, Singapore, and South Korea have almost the same ESBL rates of 39.1%, 37.5%, and 32.4%, respectively – close to that of the Philippines with 40% (Hawser *et al.* 2009). The continued rise of ESBL-producing bacteria therefore needs immediate action, especially as information about ESBL in the Philippines is limited.

It was also found that ESBL producers are almost equally prevalent in different hospitals located in Northern Luzon, Philippines. In the Philippines, there are very few studies on the prevalence of ESBL-producing *K. pneumoniae* and those published are mostly focused in members of the family Enterobacteriaceae in general. In the study of Bomasang and Mendoza (2003), it was reported that the prevalence of ESBL production among Enterobacteriaceae isolates from the Philippine General Hospital was 29.9%. It was also shown that prevalence of ESBL-producing *E. coli* and *Klebsiella* spp. in a tertiary hospital in Makati City was 20.1% (Villanueva *et al.* 2003). Meanwhile, in the study of Lucena *et al.* (2012) that evaluated the prevalence of ESBL-producing Enterobacteriaceae at Mindanao Sanitarium and a hospital in southern Philippines, it was shown that 30/583 (5.1%) were ESBL-producers. 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), while a study on the prevalence of ESBL producing Enterobacteriaceae from clinical isolates in a tertiary hospital in Bacolod City showed a relatively lower prevalence rate at 18.8% (Garcia *et al.* 2016). In

this present study, the prevalence of ESBL-producing *K. pneumoniae* in four different hospitals in Luzon ranging 16–20% was close to previous reports in different areas (Table 3). These results suggest that ESBL-producing *K. pneumoniae* are indeed present and might have already evolved in several hospitals all over the country. The differences in ESBL prevalence rate in the country prove that ESBL occurrence varies with respect to the region and time of collection. Increasing local and international travel and trade may have contributed in the movement of many of these genes. And because ESBL-producing *K. pneumoniae* can cause most hospital-acquired infections, it must therefore be constantly monitored in surveillance studies.

This is the first report on the predominance of the $bla_{\text{OXA-1}}$ type from phenotypically confirmed ESBL-positive *K. pneumoniae* isolated in the country, which is in contrast with previously reported cases. It was previously described that out of 39 ESBL-producing clinical isolates from the Philippine General Hospital, 37 (95%) possessed the $bla_{\text{CTX-M}}$ gene and was thus considered as the most predominant extended-spectrum β-lactamases among *Enterobacteriaceae* in Manila (Tian *et al.* 2010). Similarly, around 60% of all ESBL-producing *Enterobacteriaceae* at a private tertiary hospital in Southern Philippines produced the CTX-M type ESBLs (Lucena *et al.* 2010). Isolates of ESBL type $bla_{\text{CTX-M}}$ was also observed from phenotypically identified ESBL-producing *E. coli* isolates from the Philippines (Cruz and Hedreyda 2017). This result is not surprising because the CTX-M-type ESBLs is the most frequent ESBL-type worldwide. In Southeast Asia, the CTX-M series enzymes have been reported to be the most dominant ESBL-type in the region (Mendes *et al.* 2013). The CTX-M types appeared significantly in Vietnam and Thailand while India is completely dominated by $bla_{\text{CTX-M}}$ (Hawkey 2008). Furthermore, results of the prevalence of the bla_{TEM} among the ESBL-producing *K. pneumoniae* was similar to that $bla_{\text{CTX-M}}$ at 56%. TEM-1 is the most commonly encountered β-lactamase in Gram-negative bacteria, while TEM-3 is the first β-lactamase that displayed the ESBL phenotype. To date, around 90 TEM derivatives have already been described – most often found in *K. pneumoniae* and *E. coli.* In the study of Cruz and Hedreyda (2017), it was shown that plasmid-encoded bla_{TEM} was the most prevalent in ESBL-producing *E. coli* isolates in the Philippines and its result was also comparable to that of $bla_{\text{CTX-M}}$. In fact, in a recent study of Vital *et al.* (2018), the bla_{TEM} was found to be the predominant type among phenotypically confirmed ESBL-producing environmental samples of *E. coli* isolated from irrigation water in urban farms in Metro Manila.

Table 3. Total number of putative *K. pneumoniae* isolates from four different hospitals in Luzon and percentage of *K. pneumoniae* confirmed to be ESBL producers by phenotypic confirmatory disk diffusion test (PCDDT).

Hospitals	No. of ESBL producers (n=25) (%)
Batangas	4 (16)
Bulacan	4 (16)
Nueva Ecija	5 (20)
Rizal	5 (20)

*n = the number of isolates tested

Moreover, 15 out of 18 (83%) ESBL-positive *K. pneumoniae* isolates possessed the bla_{SHV} gene. The SHV-1 β-lactamase is commonly found in *K. pneumoniae* and is responsible for up to 20% of plasmid-mediated antibiotic resistance in this species (Bradford 2001). The bla_{SHV} gene can give rise to SHV variants possessing an ESBL phenotype such SHV-2, which is characterized only by a single amino acid substitution at position 238. Moreover, it was recently observed that majority of SHV-type derivatives possessed the ESBL phenotype. Cabrera and Rodriguez (2009) first reported the occurrence of SHV-12 among ESBL-producing Enterobacteriaceae from clinical isolates at the Philippine General Hospital. They concluded that $bla_{\text{SHV-12}}$ was the most dominant ESBL among the Enterobacteriaceae studied. In fact, no CTX-M type of ESBL were detected among the isolates, even if CTX-M became the most predominant ESBL gene among Enterobacteriaceae in the same hospital the following year based on a study conducted by Tian *et al.* (2010). This result confirms that occurrence and predominance of ESBL genes may vary from time to time and from one country to another (Varkey *et al.* 2014).

In this study, the $bla_{\text{OXA-1}}$ was found in all the *K. pneumoniae* isolates tested. The OXA-1 type is a narrow spectrum β-lactamase that confers resistance only to few cephalosporins. OXA-1 has been previously reported to be the most common OXA-type β-lactamase, as it is found in 1–10% of *E. coli* isolates (Paterson and Bonomo 2005). A number of OXA-type derivatives are not ESBL. However, studies showed that the $bla_{\text{OXA-1}}$ gene has frequently been found to be associated with genes encoding ESBLs. The prevalence of the bla_{OXA} gene greatly varies in different Asian countries. For instance, a study in Turkey reported a 34.6% occurrence (Nazik 2012) and a study in Taiwan reported a much lower 1.9% emergence of bla_{OXA} (Ma *et al.* 2015). Furthermore, a high occurrence of 78.33% has been reported in Riyadh, Saudi Arabia (Shibl *et al.* 2013). The $bla_{\text{OXA-1}}$ gene was also found in *K. pneumoniae* sputum culture isolate from China (Shi *et al.* 2008). Unfortunately, there are very few epidemiologic data on the geographical spread of OXA-type ESBLs and no studies of genotyping the bla_{OXA} gene have been reported in the Philippines.

Most studies on genotyping of ESBL genes in the Philippines amplified the bla_{CTX-M}, bla_{TEM}, and bla_{SHV} genes – which were reported to be the most common among *Enterobacteriaceae*. This present study detected bla_{OXA-1} among ESBL positive *K. pneumoniae* isolated from hospitals in Luzon. It is plausible therefore that the bla_{OXA-1} gene was already present from the time of sampling in the previous studies and may not yet be the predominant gene. This gene might just have spread through time due to the fast-paced transfer of β-lactamase genes observed in the last few years. The prevalence of β-lactamase genes is also believed to be attributed to the differences in the type and volume of consumption of antibiotics at different times and at different regions.

As presented in this report, majority of the ESBL-producing *K. pneumoniae* isolates harbored more than one type of β-lactamase genes. Recent studies have also shown that hyperproduction of β-lactamases may also lead to the development of resistance to β-lactamase inhibitors (Sugumar *et al.* 2014). The concomitant presence of different β-lactamases and their variants have been reported to affect β-lactam-β-lactamase inhibitor combination. This is particularly important when the OXA-1 type β-lactamases are expressed as these enzymes are weakly inhibited by clavulanate (Canton *et al.* 2008). Moreover, the association of OXA-1 with CTX-M also makes isolates resistant to β-lactam-β-lactamase inhibitor combination. In this study, the bla_{OXA-1} gene was common in all of the isolates tested, which might also be the reason why some isolates initially detected as ESBL-positive by antibiotic susceptibility testing were found negative by PCDDT. Some of these isolates might have exhibited resistance to the β-lactamase inhibitor (clavulanic acid) in combination with β-lactam antibiotics used in PCDDT. This study provides new insights in understanding the mechanism of resistance and implications of the presence of multiple β-lactamases, particularly OXA-1, to the present confirmatory methods of detecting ESBL-producers.

The high prevalence of ESBL-producing *K. pneumoniae* with multiple β-lactamases (CTX-M,TEM, SHV, OXA-1) therefore poses serious problem in the hospital settings. Thus, close monitoring and antimicrobial stewardship plays a significant role in controlling the spread of ESBL in our country. These results further strengthen the urgent need of a routine bacterial screening for ESBL in the Philippines to provide fast and accurate results.

CONCLUSION AND RECOMMENDATIONS

From a total of 100 *K. pneumoniae* isolated from four different hospitals in Luzon, 18% were confirmed to be ESBL-producers by PCDDT. Typing of the *bla* genes showed that majority the isolates contained multiple β-lactamase genes. Interestingly, bla_{OXA-1} was reported to be present in all of the isolates – suggesting that it might be the most dominant gene among the *K. pneumoniae*. Effects of OXA-1 mediated resistance to β-lactam-β-lactamase inhibitor combination should also be further evaluated. Transconjugation experiments to demonstrate horizontal gene transfer and possibly determine the location of the ESBL genes may be conducted in the future to further examine the efficiency of transmission. DNA sequencing of *bla* genes from all of the different isolates is also recommended to determine specific variants of these genes.

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