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Screening of Bacteriophages against Different Genotypes of Extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* Isolated from Five Hospitals in Cavite and Metro Manila, Philippines

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RESEARCH ARTICLE

Abstract

Background: Extended-spectrum β-lactamase (ESBL) *K. pneumoniae* infections are emerging health problems in the Philippines. Recently, bacteriophages have been explored to target several antibiotic-resistant bacteria as a potential alternative therapeutic option to conventional antibiotics.
Objectives: This study isolated extended-spectrum β-lactamase (ESBL) producing *K. pneumoniae* harboring different β-lactamase genes to evaluate the host range specificity of isolated bacteriophages.
Methodology: *K. pneumoniae* were isolated from five selected hospitals in Cavite and Metro Manila, Philippines and their ESBL-production was determined through the Phenotypic Confirmatory Disc Diffusion Test (PCDDT). The identity of the isolates was then confirmed by amplification and sequencing of the *16 rRNA* gene. The type of β-lactamase genes carried by the *K. pneumoniae* ESBL-positive strains was detected by amplification of the bla_{CTX-M}, bla_{TEM}, bla_{SHV} and bla_{OXA-1} genes. Meanwhile, bacteriophages were isolated from water samples in Marikina River and their host range specificity was tested against the different ESBL-producing *K. pneumoniae* strains.
Results: From a total of 25 *K. pneumoniae*, 6 (24%) were found to be ESBL-producers by PCDDT. Genotyping of the β-lactamase genes showed that one of the phenotypically confirmed isolates contained the bla_{CTX-M} while another possessed both the bla_{CTXM} and bla_{SHV} genes. Furthermore, another isolate harbored the bla_{CTX-M}, bla_{OXA-1}, and bla_{SHV} genes while the remaining isolates contained all the four *bla* genes tested. Meanwhile, two virulent phages namely, KP1 and KP2 that showed the largest clearing zones against *K. pneumoniae* were selected to determine their host range specificity against the different ESBL-positive *K. pneumoniae* strains. Both phages were able to infect and lyse all ESBL-positive *K. pneumoniae* regardless of the type or number of *bla* genes they possessed. Phage KP2, which showed the highest lytic capability, was then subjected to Transmission Electron Microscopy (TEM) and was found to belong to the Order *Caudovirales* under the Family *Myoviridae*.
Conclusion: This study showed that phage KP2 was host-specific to the different ESBL-producing *K. pneumoniae* harboring single or multiple *bla* genes suggesting that it might hold a great potential for possible phage therapy against ESBL-producing *K. pneumoniae* infections.

Keywords: *ESBL, β-lactamase genes, K. pneumoniae, bacteriophage, phage therapy*

Introduction

Extended-spectrum β-lactamases (ESBLs) are rapidly evolving group of β-lactamases that confer resistance to penicillin, first, second, and third generation cephalosporins and aztreonam but are inhibited by clavulanic acid [1]. In addition, ESBL-producing organisms exhibit co-resistance to other classes of antibiotics thus resulting in limited therapeutic options. Numerous types of ESBLs have been detected in various bacterial organisms but recent studies have shown that these enzymes are most frequently found in *Klebsiella pneumoniae* with a steady increase [2,3]. As opportunistic pathogens, *Klebsiella* species primarily attack hospitalized patients who are immunocompromised and those suffering from severe underlying diseases including bacteremia, pneumonia, urinary tract, surgical wounds, and several other types of human infections. ESBL-producing *K. pneumoniae*

have been reported to cause majority of nosocomial outbreaks and are responsible for at least 10% of all hospital-acquired infections [4]. The inability to recognize ESBL-producers may result to incorporating treatment failure in patients who receive inappropriate antibiotics and increase of multidrug-resistant pathogens which may require costly control efforts [5,1].

In recent years, extended-spectrum β-lactamase in *Enterobacteriaceae* has increasingly been reported worldwide with prevalence occurring at varying frequencies. In Southeast Asia, the prevalence of ESBL-producing *Klebsiella* was reported at 46.7% with the highest rate occurring among Indonesian isolates [6]. In the Philippines, an increase was observed in the prevalence of ESBL-producing *K. pneumoniae* ranging from 10-43% from 1999 to 2012 [7]. The prevalence of ESBL production among *Enterobacteriaceae* isolates at the Philippine General Hospital (PGH) was reported to be 29.9% while ESBL-producing *E. coli* and *Klebsiella* at the Makati Medical Center was reported at 20.1% [8,9]. Moreover, the study of Lucena *et al*. [10] reported the prevalence of ESBL-producing *Enterobacteriaceae* at Mindanao Sanitarium and a tertiary hospital in Southern Philippines to be 5.1%. A study on ESBL production in clinical isolates of *E. coli* from three hospitals in Luzon showed a prevalence of around 22.6%, while ESBL-producing *Enterobacteriaceae* from clinical isolates in a tertiary hospital in Bacolod City showed a prevalence of 18.8% [11,12]. The Antimicrobial Resistance Surveillance Program (ARSP) of the Department of Health for 2018 showed that the ESBL production cumulative rate of *K. pneumoniae* in the Philippines was 58% which was relatively higher than the cumulative rate of 41% in the previous year [13].

Extended-spectrum β-lactamases mainly arise due to mutations in β-lactamases encoded by the *bl*a genes and more than 300 different ESBL variants have already been described [14]. The TEM and SHV-type are the most common plasmid-mediated β-lactamases that are often found in *E. coli* and *K. pneumoniae* and most ESBL's are derivatives of the TEM and SHV enzymes [15]. TEM-1 is commonly encountered in Gram-negative bacteria and the TEM-3 derivative is the first TEM-type β-lactamase that displays ESBL phenotype. To date, around 90 TEM derivatives have already been discovered. The SHV-1 β-lactamase, on the other hand, is responsible for up to 20% of plasmid-mediated antibiotic resistance in *K. pneumonia* [15]. Majority of SHV-type derivatives possessed the ESBL phenotype. Meanwhile, a plasmid-mediated CTX-M ESBLs that preferentially hydrolyze cefotaxime have begun to emerge in many countries during the past decade and are now considered the most frequent ESBL-types worldwide [16]. In Southeast Asia, the CTX-M series enzymes have also been reported to be the most dominant ESBL-types in the region [17,18]. Lastly, the OXA-type is another growing family of ESBLs originally discovered in *P. aeruginosa* but was found in 1-10% of *E. coli* isolates [1]. Although a number of OXA-type derivatives are not ESBL, studies showed that the $bla_{\text{OXA-1}}$ gene is frequently associated with genes encoding ESBLs. Normally, carbapenems are often the preferred antimicrobial agent for the treatment of serious infections caused by ESBL-producing organisms and are considered to be the reliable last resort treatment for ESBL bacterial infections. However, the spread of carbapenem-resistant bacteria has also recently been reported [19]. Carbapenem resistance is another ongoing public health problem of global dimension and the emergence of carbapenem-resistant *K. pneumoniae* in the Philippines also poses a potential health threat. There is therefore a need for efficient infection control practices and intervention strategies to reduce further selection and spread of these increasingly resistant pathogens.

The search for alternative therapy to conventional antibiotics has become an important research avenue given the increasing number and types of extended-spectrum β-lactamase bacterial infections especially within hospital settings. Prior to the discovery and widespread use of antibiotics, it was already suggested that bacterial infections could be prevented and/or treated by the administration of bacteriophages [20]. Bacteriophages constitute a group of viruses that can specifically infect and lyse bacteria while not affecting cell lines from other organisms. The initial fervor over phage therapy as a treatment for bacterial diseases in the pre-antibiotic era was already enormous. The concept of therapeutic use of phages to treat bacterial infection, however, was highly controversial and was not widely accepted by the public or medical community alike. Recently, because of the emergence of multi-drug resistant bacteria, there has been a renewed interest at phage therapy as a new and potentially viable treatment option for difficult-to-treat bacterial infections [21]. Bacteriophages are considered to be good candidates because of their high specificity owing to narrow host ranges and mode of action which is unrelated to antibiotic targets. It also has self-propagating and self-limiting activities facilitating low dosing [22]. Unlike broad-spectrum antibiotics, phages are highly specific and do not elicit resistance from untargeted bacterial strains [23]. Bacteriophages could prove to be superior to antibiotics because they are persistent, inactive, and non-pathogenic outside their bacterial host. Compared to conventional therapeutic approaches, phage therapy could be more effective in treating emerging

antibiotic-resistant pathogenic strains of bacteria. The objectives of this study therefore, were to isolate , identify, and genotype ESBL-producing *K. pneumoniae* carrying single or multiple β-lactamase (*bla*$_{CTX-M}$, *bla*$_{OXA}$, *bla*$_{SHV}$, and *bla*$_{TEM}$) genes. In addition, the study aimed to isolate and characterize lytic bacteriophages against ESBL-producing *K. pneumoniae* in an effort to develop new possible therapeutic interventions to control infection of these pathogens.

Methodology

Sample Collection

Bacterial samples were collected from five different tertiary hospitals in Cavite Province (specifically in the cities of Bacoor and General Trias) and in Metro Manila (specifically in the cities of Las Piñas, Marikina, and Quezon City). The hospitals were chosen because the isolation and genotyping of ESBL-producing bacteria in these areas have not been reported previously. Sample collection was obtained with the permission of the heads of the respective hospitals following specific ethics committee guidelines of the hospitals and Miriam College, Quezon City. Isolation of bacterial strains was done by either swabbing the surfaces with pre-moistened swabs in Phosphate buffer or directly exposing *Klebsiella* Selective Agar (KSA) plates in different areas of the hospitals such as the pharmacy and information desk counters, emergency room beds and tables, public comfort rooms, and oxygen masks. All the collected samples were stored in a thermal cooler and immediately transported to Miriam College Microbiology Research Laboratory. Plates were then immediately incubated for 24 h at 37° C and colonies that grew in KSA medium were randomly selected, purified in Nutrient agar (NA), and partially characterized by conventional morphological and biochemical tests.

ESBL-producing *K. pneumoniae* BUL_ICUVent as positive control for genotyping was previously isolated from a hospital in Bulacan, Philippines. This isolate was confirmed to be ESBL-producing by both antimicrobial susceptibility testing and PCDDT and to possess, the *bla*$_{CTX-M}$, *bla*$_{OXA}$, and *bla*$_{TEM}$ genes by PCR [24]. Negative controls of non-ESBL-producing *K. pneumoniae*, *Bacillus cereus*, and *Escherichia coli* were all obtained from the Miriam College Culture Collection, Quezon City.

Screening of ESBL production

All isolates that initially displayed morphological and biochemical characteristics of *K. pneumoniae* were further screened for the production of extended-spectrum β-lactamases (ESBLs) using the Phenotypic Confirmatory Disc Diffusion Test (PCDDT) on Mueller Hinton Agar (MHA) as recommended by the National Committee for Clinical Laboratory Standards [25]. Two to three colonies were picked up from NA plates, inoculated in Luria Bertani (LB) broth and incubated at 37ºC for 16 to 18 h. The bacterial cultures were adjusted with saline solution until turbidity 0.5 McFarland standard was reached [26]. The suspensions were swabbed onto the surface of two MHA plates and 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 both plates were incubated for 16-18 h at 37ºC. An increase in the inhibition zone diameter of ≥ 5 mm for either antimicrobial agent tested in combination with clavulanic acid versus ceftazidime or cefotaxime discs alone was considered for ESBL-production.

Amplification and sequencing of 16s rRNA gene

The identity of the ESBL-positive isolates was confirmed through the amplification of the 16S rRNA gene by Polymerase Chain Reaction (PCR) using Quanta SI-96 Thermocycler. First, the genomic DNA was extracted using GF-1 Genomic DNA Extraction Kit (Vivantis, Malaysia) and used as a template for PCR amplification. The 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 [27]. A total of 50 µL of PCR mixture was used with the following components: 25 µL Taq Master Mix; 1 µL of 10 µM 16S rRNA forward primer; 1 µL of 10 µM 16S rRNA reverse primer; 1 µL of 50 mM MgCl; 20 µL nuclease-free H2O, and; 2 µL extracted bacterial DNA template. The PCR conditions used were as follows: a) initial denaturation at 95°C for 5 min; b) 35 cycles of denaturation (30 sec at 95°C) annealing (30 sec 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 by Agarose Gel Electrophoresis (AGE) in a 1.5% gel at 100 volts for 30 min. Amplicons were sent to Asiagel Corporation, Quezon City for DNA sequencing. Gene sequences were aligned from nucleotide database using Basic Local Alignment Sequencing Tool (BLAST) and the % homologies and possible identities were determined.

Detection of bla$_{CTX-M}$, *bla*$_{OXA}$, *bla*$_{SHV}$, *bla*$_{TEM}$ *genes*

The type of the β-lactamase genes carried by the different phenotypically confirmed *K. pneumoniae* isolates was determined by amplification of the *bla*$_{CTX-M}$, *blaO*$_{XA-1}$, *bla*$_{SHV}$

and *bla*$_{TEM}$ genes. The forward and reverse primers used for the amplification of these genes and their respective amplicons are shown in Table 1. The reaction mixture of 50 µL was used in all of the genes detected with the following components: 25 µL of Taq Master Mix, 1 µL of 50 mM MgCl, 20 µL nuclease-free H2O, 2 µL of the extracted genomic DNA, and 1 µL of 10 µM forward and reverse primers. The PCR conditions used for the amplification of the *bla*$_{CTX-M}$ and *bla*$_{OXA-1}$ genes were: 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); while for the *bla*$_{SHV}$: 94°C for 4 min (initial denaturation), 35 cycles of denaturation at 94°C for 1 min, 57.5°C for 20 sec (annealing), 72°C for 30 sec (extension), and 72°C for 5 min (final extension). Lastly, for the *bla*$_{TEM}$: 94°C for 4 min (initial denaturation), 35 cycles of denaturation at 94°C for 1 min, 59°C for 20 sec (annealing), 72°C for 30 sec (extension), and 72°C for 5 min (final extension).

The resulting PCR products were analyzed by electrophoresis using 1.5% agarose gel for 30 minutes at 100 volts. The gels were stained with Gel red and band was observed at desired position and photographed on an ultraviolet light transilluminator (BIO-RAD Gel Doc EZ Imager). A molecular weight standard (1 kb ladder) was included on each gel. A representative isolate containing the genes of interest was sent to Asiagel Corporation, Quezon City to confirm the correct identity of the amplified genes. The gene sequences were then aligned with the known sequences in the PUBMED nucleotide database using the Basic Local Alignment Search Tool (BLAST).

Bacteriophage Isolation and Purification

Bacteriophages were isolated from water samples collected from Marikina River, Metro Manila, Philippines. A one hundred mL water sample was taken from 3 stations along the river and centrifuged at 10,000 rpm for 5 min. The supernatant was subjected to successive ultrafiltration using 0.20 µm pore size syringe filters. For the pre-enrichment, 30 µL of the filtered sample and 100 µl of the 6 h-8h *K. pneumoniae* host strain broth culture were mixed with 30 mL of Tryptic Soy Broth (TSB). The culture was incubated at 37°C overnight and centrifuged at 10,000 rpm for 10 min at 40°C. The phage lysate was again filtered and stored in sterile screw-capped tubes. Serial dilution (1:10) of the phage lysates was conducted using Phosphate Buffer Saline (PBS) solution as diluent. The Spot test method was used to analyze the presence of phages that can lyse the bacterial host strain of *K. pneumoniae* lawned onto Tryptic Soy Agar (TSA).

The Double-layer Plaque Technique was then performed for the purification of bacteriophage. One hundred µL of the phage lysate was inoculated to 100 µL of 6-8 h culture of *K. pneumoniae* then mixed into 0.6% soft TSA and the mixture was then poured over pre-plated TSA plates. After solidification, plates were inverted and incubated at 37°C overnight. Individual plaque formation was observed at different dilutions and phages exhibiting formation of plaques or zones of clearance were selected. Clear plaques showing the largest sizes were individually picked up using a sterile microneedle and placed in 2 mL of TSB. This was shaken and centrifuged at 10,000 rpm for 2 min and supernatant was filtered using 0.20 µm pore size syringe filter. An aliquot of 100 µL of the obtained phage lysate was mixed with 100 µL of 6-8 h bacterial culture in 3 mL of 0.6% soft TSA and the mixture was immediately poured onto TSA plates. After solidification, plates were inverted and incubated at 37°C overnight.

Phage Propagation

For the propagation of phages, the bacterial host of *K. pneumoniae* was grown in 10 mL TSB at 370°C until log phage

Table 1. *Summary of forward and reverse primers used for the amplification of different bla genes and their expected sizes.*

Gene	Forward Primer (5 →3')	Reverse Primer (5'→3')	Expected Size (bp)	Reference
bla$_{CTX-M}$	5'-SCS ATG TGC AGY ACC AGT AA-3'	5'-ACC AGA AYV AGC GGB GC-3'	585	[28]
bla$_{TEM}$	5'-GCT CAC CCA GAA ACG CTG GT-3'	5'-CCA TCT GGC CCC AGT GCT GC-3'	686	[28]
bla$_{SHV}$	5'-CCC GCA GCC GCT TGA GCA AA 3'	5'-CAT GCT CGC CGG CGT ATC CC-3'	733	[28]
bla$_{OXA-1}$	5′-CTG TTG TTT GGG TTT CGC AAG-3′	5′-CTT GGC TTT TAT GCT TGA TG-3′	519	[29]

* (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.

was reached (6-8 h) and inoculated with 5 mL of purified phage lysates in 85 ml TSB. After 24 h of incubation, the culture subjected to centrifugation at 7500 rpm for 15 min was then filtered with a 0.20 µm syringe filter. The phage suspension was subjected to serial dilution then 100 µL phage dilution was mixed with 250 µL 6-8 h old *K. pneumoniae* in 3 mL of 0.6 % soft TSA. This was repeated for the rest of the dilutions. The soft agar tubes were plated on TSA plates and were incubated overnight at 37°C. The plaque-forming units per milliliter (PFU/mL) was then computed.

Host Range Specificity

The isolated and purified virulent phages were investigated for their host range specificity against the different types of ESBL-producing *K. pneumoniae* containing single or multiple *bla* genes. Purified phages were inoculated to these different strains of ESBL-producing *K. pneumoniae* as host cells to check whether these phages have the ability to lyse the different bacteria. The different ESBL-producing bacterial isolates were pour plated on TSA plates and 10 µL of purified phages were spotted onto the bacterial lawns. After 18-22 h of incubation, spot clearing on permissive strains and bacterial lawns were observed and evaluated based on lysis efficiency classified using the system employed by Kutter [30].

Examination of Phage Morphology

The morphology of the ESBL-*K. pneumoniae* specific phage was observed by Transmission Electron Microscopy (TEM) at the Research Institute for Tropical Medicine (RITM), Alabang, Muntinlupa. A volume of 1.5 mL of the purified lysates was sent to RITM for TEM services for visualization of the purified phage morphology. Negative staining using 2% uranyl acetate (pH 4.0) was performed. The classification of phage based on morphology and size was measured by nanometers (nm).

Results and Discussion

Characterization of K. pneumoniae isolates

K. pneumoniae were isolated from fomites and air samples from five selected hospitals in Bacoor Cavite, General Trias Cavite, Las Piñas, Marikina, and Quezon City. A total of 25 isolates of *K. pneumoniae* consisting of five isolates from each hospital were randomly selected. These isolates were initially screened based on growth characteristics in *Klebsiella* Selective Agar (KSA) which contains a chromogenic substrate that is cleaved specifically by *Klebsiella* species to produce a purple-magenta colored colony (Fig. 1A). Selected isolates that grew on KSA plates were then purified and further characterized based on standard microbiological techniques.

In this study, *K. pneumoniae* was actively selected because it is one of the most medically important causes of nosocomial infections and the most frequently isolated Gram-negative bacteria in hospital settings. *K. pneumoniae* belong to the

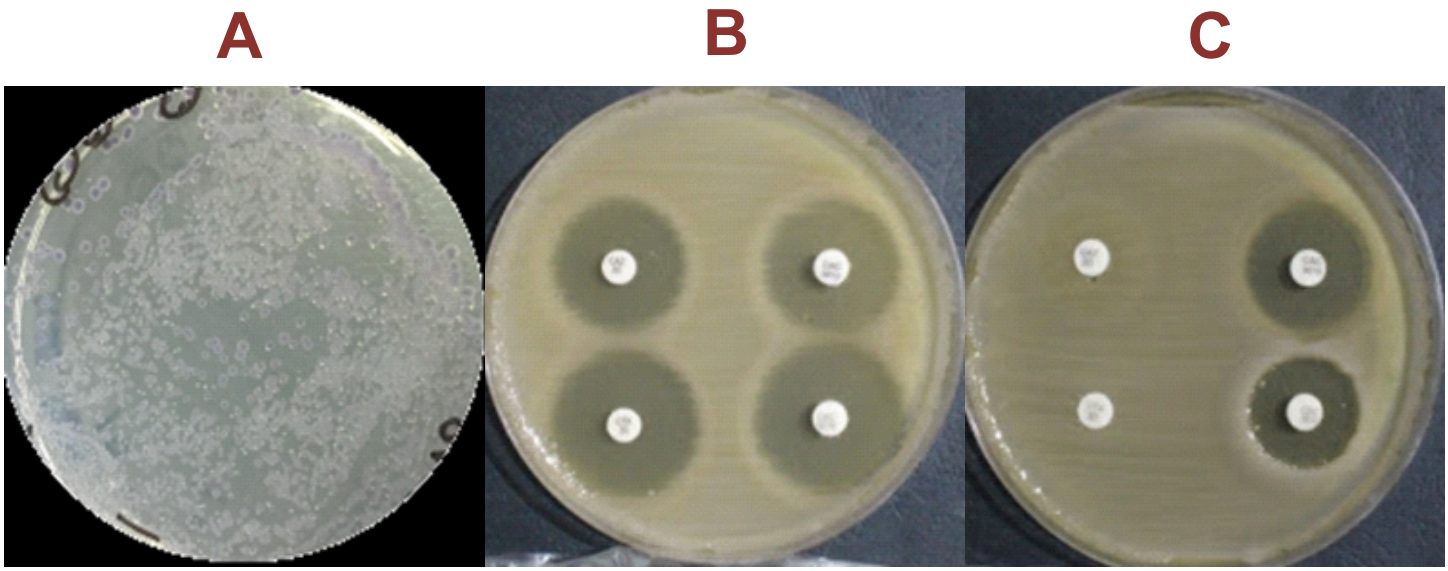

Figure 1. *Isolation of environmental samples of K. pneumonia and PCDDT used for the screening of ESBL-production. A: growth of putative K. pneumonie on KSA plates; B: PCDDT of non-ESBL-producing strain and; C: ESBL-positive isolates tested against antibiotic discs containing cefotaxime (CTX), cefotaxime-clavulanic (CXV), ceftazidime (CAZ) and ceftazidime-clavulanic (CZV).*

normal intestinal flora of man and is harmless, but in people with weakened immune systems or is being weakened by other infections, this isolate can occur as a potential pathogen. *Klebsiella* is an opportunistic pathogen that can give rise to bacteremia, pneumonia, tract infection, and several other types of human infections. Recently, it has been reported that there is an increase in *Klebsiella* infections particularly in hospital settings due to strains that produce extended-spectrum β-lactamases (ESBL) [1]. These ESBL-producing *K. pneumoniae* have gradually emerged in the last decades and their prevalence have increasingly reached alarming rates. Infections caused by ESBL-producing pathogens have often resulted in limited therapeutic options that caused treatment failures. In the Philippines, the resistance pattern of ESBL-producing *K. pneumoniae* has been increasing consistent with findings of global patterns [13].

Screening of ESBL production

Of the 25 putative *K. pneumoniae* isolates, 6 (24%) were confirmed positive for the production of extended-spectrum β-lactamase (ESBL) enzymes as determined by the Phenotypic Confirmatory Disc Diffusion Test (PCDDT). This test consists of measuring the growth-inhibitory zones around both cefotaxime (CTX) and ceftazidime (CAZ) disks with or without clavulanate (CA) and has been routinely used to detect ESBL strains of *K. pneumoniae* and *Escherichia coli* [31]. An increase of at least 5 mm in the growth inhibitory zone diameter around either cefotaxime (CTX) and cefotaxime-clavulanic (CTX-CXV) or ceftazidime (CAZ) and ceftazidime-clavulanic (CAZ-CZV) antibiotics is considered ESBL-positive as shown in Fig. 1C. Two of the ESBL-positive isolates were from a hospital in Cavite (CERch, CERcr) while four were isolated from a hospital in Marikina (MWDb, MWDm, MERb, MWDd) (Table 2). ESBL-positive *K. pneumoniae* were not detected from the other hospitals sampled probably because environmental strains of ESBL-producing *K. pneumoniae* were not present or might have not been randomly selected on the KSA plate. In this study, the prevalence of ESBL-producing *K. pneumoniae* was not determined per hospital because it only aimed to isolate *K. pneumoniae* of different genotypes for bacteriophage host range specificity studies. The phenotypically confirmed ESBL-producing putative *K. pneumoniae* isolates were then used for molecular identification and genotyping of the β-lactamase genes.

Genotypic characterization and typing of β-lactamase genes

The identities of the ESBL-producing isolates were confirmed by the amplification and sequencing of the 16S rRNA gene and alignment of DNA sequences from nucleotide database showing the highest sequence homology to *K. pneumoniae*. The type of β-lactamase genes carried by these ESBL-positive *K. pneumoniae* strains was then determined by PCR. ESBL enzymes that provide resistance against β-lactam antibiotics are encoded by the *bla* genes and the amplification of these genes provide a good confirmatory test to indicate ESBL-production. This study focused only on bla_{CTX-M}, bla_{OXA}, bla_{SHV}, bla_{TEM} genes because based on reports, these are

Table 2. *Results of PCDDT and summary of the different β-lactamase genes detected from the ESBL-producing K. pneumoniae isolates from selected hospitals in Bacoor Cavite, General Trias Cavite, Las Piñas, Marikina, and Quezon City.*

Sample code	PCDDT	bla_{CTX-M}	bla_{OXA-1}	bla_{SHV}	bla_{TEM}	
K. pneumonia	-	-	-	-	-	P
E. coli	-	-	-	-	-	C
K. pneumoniae BUL_ICUVent	+	+	+	-	+	D
K. pneumoniae (CERch)	+	+	-	-	-	D
K. pneumoniae (MWDb)	+	+	-	+	-	T
K. pneumoniae (CERcr)	+	+	+	+	-	
K. pneumoniae (MWDm)	+	+	+	+	+	(
K. pneumoniae (MERb)	+	+	+	+	+	+
K. pneumoniae (MWDd)	+	+	+	+	+	)

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. K. pneumoniae BUL_ICUVent positive control was previously isolated from a hospital in Bulacan and confirmed to be ESBL-producer possessing the bla_{CTX-M}, bla_{OXA}, and bla_{TEM} genes (Cornista *et al.*, 2019).

considered to be the most commonly occurring β-lactamase genes among members of the family *Enterobacteriaceae* [32]. The *bla* genes are commonly located on transferable elements such as plasmids or transposons that can facilitate horizontal gene transfer of antibiotic resistance among bacterial strains. The detection of the type of *bla* genes carried by the ESBL-producing *K. pneumoniae* by molecular method provides useful information about its epidemiology and risk factors associated with these infections. However, PCR method will not discriminate against the different variants of the β-lactamase genes but will just aid in the detection and differentiation of these genes. In this study, variants of the different amplified *bla* genes were also not determined. DNA sequencing was conducted just to confirm the correct identity of the amplified *bla* genes from the different *K. pneumoniae* strains.

Results of this study showed that all the ESBL-positive *K. pneumoniae* harbored the bla_{CTX-M} gene (Table 2). The bla_{CTX-M} has been reported in several parts of the world as the predominant ESBL genotype in *Enterobacteriaceae* among community-acquired and nosocomial infections [33]. The bla_{CTX-M} gene was previously found to be the most predominant ESBLs among *Enterobacteriaceae* at the Philippine General Hospital [34]. Moreover, the CTX-M type ESBL-producing *Enterobacteriaceae* was also found to be the predominant ESBL gene in a private tertiary hospital in Southern Philippines [10]. On the other hand, 5 out of 6 ESBL-positive *K. pneumoniae* isolates (83.3%) in this study contained the bla_{SHV} gene (Table 2). The SHV-1 β-lactamase has been reported to be most commonly found in *K. pneumoniae* and in many strains the $blaS_{HV-1}$ can be integrated into the bacterial chromosomes [15]. Cabrera and Rodriguez first reported the occurrence of SHV-12 as the most dominant among ESBL-producing *Enterobacteriaceae* from clinical isolates at the Philippine General Hospital [35].

Furthermore, this study showed that 4 out of 6 (66.7%) of *K. pneumoniae* strains carried the bla_{OXA-1} gene (Table 2). The bla_{OXA-1} has been previously found in plasmid and integron locations in large variety of gram-negative bacteria. The OXA-type β-lactamases confer resistance to ampicillin and cephalothin and are characterized by high hydrolytic activity against oxacillin and cloxacillin but are poorly inhibited by clavulanic acid [15]. To date, very few data on the prevalence of OXA genes among ESBL-producing *K. pneumoniae* have been reported in the Philippines. But in a recent study of Cornista *et al.*, [24] it was shown that the bla_{OXA-1} gene was the most predominant among the ESBL-producing *K. pneumonia* isolated from 4 provincial hospitals in Luzon, Philippines. Lastly, 3 out of 6 (50%) ESBL-producing *K. pneumoniae* isolate harbored the bla_{TEM} gene. Although TEM-type β-lactamases are most often found in *E. coli* and in strains of *K. pneumoniae*, they are also found in other species of gram-negative bacteria with increasing frequency. Cruz and Hedreyda have shown that plasmid-encoded bla_{TEM} was found to be the most prevalent among ESBL- producing *E. coli* isolates in the Philippines which was comparable to that of the bla_{CTX-M} gene [36].

Results of this study also showed that majority of the ESBL-producing *K. pneumoniae* isolates harbored more than one type of ESBL gene. *K. pneumoniae* (MWDb) contained both the bla_{CTX-M} and bla_{SHV} genes while *K. pneumoniae* (CERcr) harbored the bla_{CTX-M}, bla_{OXA}, and bla_{SHV} genes. But it was alarming to note that ESBL *K. pneumoniae* (MWDm, MERb, MWDd) strains contained all the four *bla* genes monitored in this study (Table 2). Infection with this type of ESBL *K. pneumoniae* might provide a potential threat to patients as it will be more difficult to manage within a hospital setting. It seems that among all the ESBL genes, the CTX-M was the most common as it was present in all ESBL positive strains tested followed by the SHV present in five and OXA-1 in four of the isolates. The TEM was shown to be the least common as it was only found in three of the six ESBL-positive isolates. Moreover, the presence of multiple genes in ESBL-*K. pneumoniae* isolates found in this study suggest that further care on the administration of antibiotics must be done by our health care providers. It also strengthens the need for a routine bacterial screening of ESBL-producers before prescribing antibiotics especially in a country like the Philippines where poor monitoring of antibiotic resistance is observed.

Bacteriophage Isolation and Purification

In response to the global emergence of multiple antibiotic resistance, there has been a renewed interest in bacteriophage as potential treatment for several bacterial infections. Bacteriophages have been found to be effective against a wide variety of pathogenic bacteria thus, have been considered a possible alternative to conventional antibiotics. Phages that are host-specific against a wide spectrum of bacteria-resistant antibiotics may have significant potential as novel therapeutic agents. This study isolated bacteriophages that were effective against previously isolated ESBL-producing *K. pneumoniae* strains harboring different types and numbers of β-lactamase genes.

Water samples were collected from the Marikina River located in Eastern Metro Manila which is a tributary to the Pasig River with headwater located in Sierra Madre

Mountains in Rodriguez, Rizal, Philippines. Because of rapid urban development and consequently excessive and indiscriminate discharge of wastes, the Marikina River has been considered to be highly polluted.

This river, therefore, is suitable for the isolation of bacteriophages which are abundant in polluted environments and are considered to be the most ubiquitous entities on earth [37]. In fact, general and host-specific bacteriophages have been comprehensively tested as potential indicators of faecal pollution and have been useful in pollution assessment and control in polluted waters.

In this study, water samples from the river were first screened for the presence of phages by enrichment in Tryptic Soy Broth (TSB) with host cells of non-ESBL *K. pneumoniae* to allow a higher concentration of phage lysate. Enrichment allowed initially a small population of the desired phages to propagate until they reach a concentration in the culture which is easily detected by routine plating methods [38]. The phage filtrate was then spotted onto lawned culture of the host bacterium and resulted in clear zones indicating initial approximation of its lytic ability against *K. pneumoniae*. The plaques all appeared to be clear, suggesting that the isolated phages were virulent (Fig. 2).

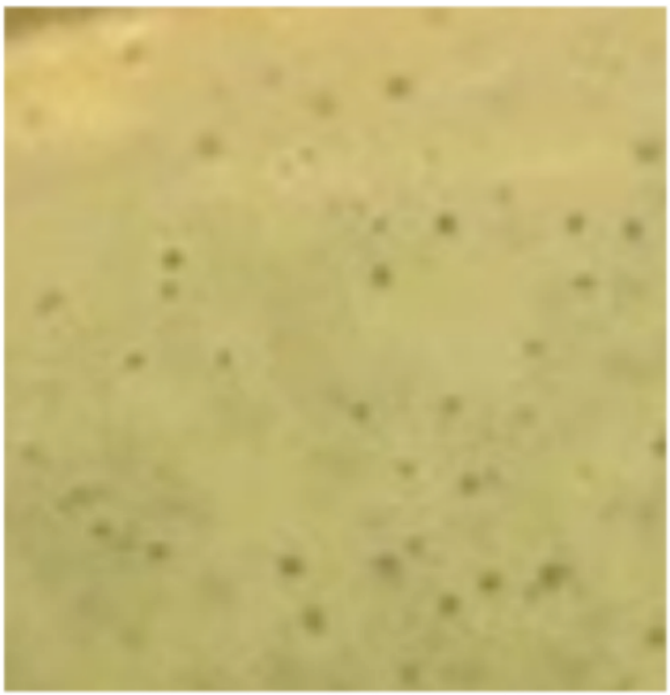

Figure 2. *Double Agar Overlay of lytic phage and K. pneumoniae on 0.6% soft TSA plates. Phages that showed the largest plaque size were chosen and purified.*

The purification of bacteriophage was performed through the Double-layer Plaque Technique and two phages named KP1 and KP2 that showed the largest clear plaque zones were selected and further purified. The plaque size is one of the criteria normally used in selecting possible bacteriophages for phage therapy since as plaque size increases, the more cells are lyzed. This study also used plaque size as a criterion in selecting phages for infection of isolated ESBL-producing *K. pneumoniae* strains. The plaques were viewed under the microscope and the plaques sizes were measured to be 0.55 mm for KP1 and 0.72 mm for KP2 (Table 3). The measured plaque sizes of the purified phages that ranged from 0.30 mm to 0.80 mm have already been previously used for possible phage therapy [39].

Phage Propagation

Bacteriophages require the metabolic activity of suitable host cells in order to propagate. The basis of traditional phage assay involves the interaction of a single lytic phage particle and a permissive bacterium, which results in the host bacterium's lysis and the release of newly formed phage progeny. To quantify phages in a given lysate, the Plaque Assay was used for tittering the phages and the plaque-forming units (PFU) were calculated. A confluent monolayer of host *K. pneumoniae* cells was infected with serially diluted KP1 and KP2 virions. An immobilizing overlay medium prevents the spreading of viral infection and restricting the viral replication within the monolayer. The infected cells then continued the replication-lysis-infection cycle and eventually resulted in the formation of distinct plaques [40]. The dissemination of viral progenies in all directions during infections of the embedded host cells makes the plaques circular [41,42].

In this study, the phage titer for KP1 was computed as 5.7 x 109 while KP2 was found to be 4.1 x 109 (Table 3). The production of high phage titer of a given lysate is necessary since titre of phages significantly decreases when administered in phage therapy [43]. In this study, both KP1 and KP2 were found to contain high concentration of phages based on their phage titer and thus, were both used for the host specificity studies.

Host Range Specificity

The host range specificity is a very important characteristic that makes bacteriophages a potential therapeutic agent against bacterial infections. In this study, the host range of the bacteriophages KP1 and KP2 was tested

to determine its genus specificity and whether it could infect the different ESBL-positive *K. pneumoniae* harboring different types of β-lactamase genes. Host range was analyzed by spotting the phage lysates onto lawns formed by the different genotypes ESBL-positive *K. pneumoniae* containing single or multiple *bla* genes. Four of the ESBL-producing *K. pneumoniae* strains were used for host range specificity since some strains contained the same genotype thus, only one representative from this strain was tested. After 18-22 h of incubation, plaques or zones of lysis were observed. Results showed that the two virulent phages tested were able to infect all the host cells of *K. pneumonia* containing different ESBL genes (bla_{CTX-M}, bla_{OXA-1}, bla_{SHV}, and bla_{TEM}) as indicated by clear plaque zones of lysis (Fig. 3). This proves that these phages infect ESBL-*K. pneumoniae* regardless of the type or the number of *bla* genes present. Both phages were also seen to exhibit lytic activity against non-ESBL *K. pneumoniae* because this host belongs to the same genus. In contrast, the phages were not able to cross infect and did not show lytic activity against other bacterial hosts such as *E. coli* and *Bacillus cereus*. These results indicate that the phages have host specificity and do not possess a broad host range activities. The fact that these isolates have lytic activity even against *K. pneumoniae* that possessed multiple *bla* genes may indicate its significant therapeutic potential. Previous studies have already shown that bacteriophages can be effective against a wide variety of pathogenic bacteria as they can be highly host-specific to *E. coli*, *Salmonella typhi*, and *Pseudomonas aeruginosa* [44]. Bacteriophages were also found to have comparable activity to antibiotics on their rate of infection on *K. pneumoniae* and *P. aeruginosa* [45]. Bacteriophage was also applied to control the spread of pathogens such as Shigella in contaminated waters [46]. In fact, ESBL *E. coli* was also found to be susceptible to laboratory-isolated bacteriophages [22].

Several studies have revealed that bacteriophage infections are initiated when the phage interacts with the host cell surface receptor molecules [44]. Bacteriophages are known to be highly specific for their receptors, thus, show very little or no interaction with other receptors with even a slight difference in structure. This host specificity forms the basis for numerous phage typing methods used in the identification of different bacterial species or subspecies. In this study, the two bacteriophages tested were able to infect all the *K. pneumoniae* regardless of the type or number β-lactamase genes present indicating that phage susceptibility of the different ESBL-positive strains is not dependent on the type or number of *bla* genes present. There was no relationship between the presence of different *bla* genes receptors and phage susceptibility. It is plausible that the phages recognize cell surface receptors that are genus-specific since they were also able to infect non-ESBL strain of *K. pneumoniae*.

Table 3. *Computed phage titers and plaque sizes of Phage KP1 and KP2 isolated from the water sample in Marikina river.*

PHAGE	Phage Titer (PFU/mL)	Plaque Size (mm)
KP1	5.7 x 109	0.55
KP2	4.1 x 109	0.72

Results of this study also showed that among the two phages, KP2 was observed to be more permissive against non-ESBL and all ESBL-positive *K. pneumoniae* isolates harboring different *bla* genes as indicated by a larger zone of clearing. KP2 being highly specific against *K. pneumoniae* could therefore be potentially used as phage therapy against infections of ESBL-positive *K. pneumoniae*.

Examination of Phage Morphology

The morphological characteristic of the phage KP2 was then analyzed by Transmission Electron Microscopy (TEM). This provides an important step in characterization since classification of phages is mostly based on morphology. Based on morphological characteristics of the phage, it was revealed that KP2 belonged to Order *Caudovirales* since they are tailed phages (Fig. 4). The Order *Caudovirales* is classified into three families namely *Myoviridae* with long contractile tails, *Siphoviridae* with long noncontractile, tails and *Podoviridae* with short tails [47]. The tail of the phage isolates was contractile, rigid, long, and thick indicating that they might belong to the family *Myoviridae* (Fig. 4). Phages belonging to this family have tails consisting of a neck, a contractile sheath, and a central tube, as described by *Ackermann* [47]. Tail sheaths of *Myoviridae* phages contract upon infection of a bacterial host and drive the tail tube through the outer membrane creating a channel for viral genome delivery [48].

Conclusion

Extended-spectrum β-lactamase (ESBL) positive strains of *K. pneumoniae* were isolated from different areas of five hospitals located in Cavite, Las Pinas, Marikina, and Quezon City, Philippines. Genotyping of the bla_{CTX-M}, bla_{SHV}, bla_{TEM}, and bla_{OXA-1} genes showed that the different *K. pneumoniae* strains possessed single or multiple β-lactamase genes with three strains containing all the four *bla* genes tested. On the

other hand, bacteriophages KP1 and KP2 were isolated from the Marikina River and were found to infect non-ESBL and all ESBL-positive *K. pneumoniae* isolates regardless of the type and number of *bla* genes present. These phages were found to be genus specific since they were not able to infect *E. coli* and *Bacillus cereus*. Bacteriophage KP2 which showed higher lytic capability was found to belong to the Order *Caudovirales* and Family *Myoviridae*. This phage therefore holds a potential promise in phage therapy against nosocomial and secondary infections caused by ESBL-producing *K. pneumoniae*. This study provided an initial screening of bacteriophages against ESBL-positive *K. pneumoniae*. Optimal multiplicity of infection (MOI) and *in vitro* killing curves are recommended to further assess the lytic activity of the isolated phages. The determination of the efficiency of plating experiments is also recommended to compare the lytic activity of the phages across the different ESBL-producing *K. pneumoniae* isolates.

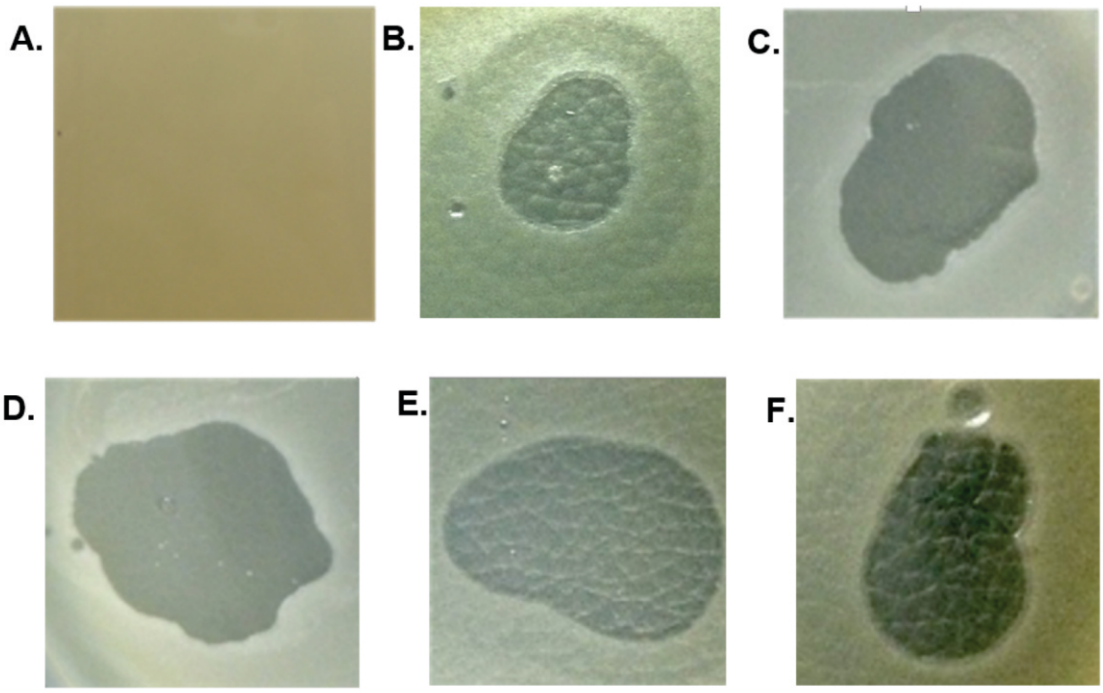

Figure 3. *Host range specificity of KP2 against non-ESBL and different ESBL-producing K. pneumonia isolates A: E. coli (control); B: non-ESBL K. pneumonia; C: ESBL K. pneumoniae (CERch); D: ESBL K. pneumoniae (MWDm); E: ESBL K. pneumoniae (CERcr); F: ESBL K. pneumoniae (MWDb).*

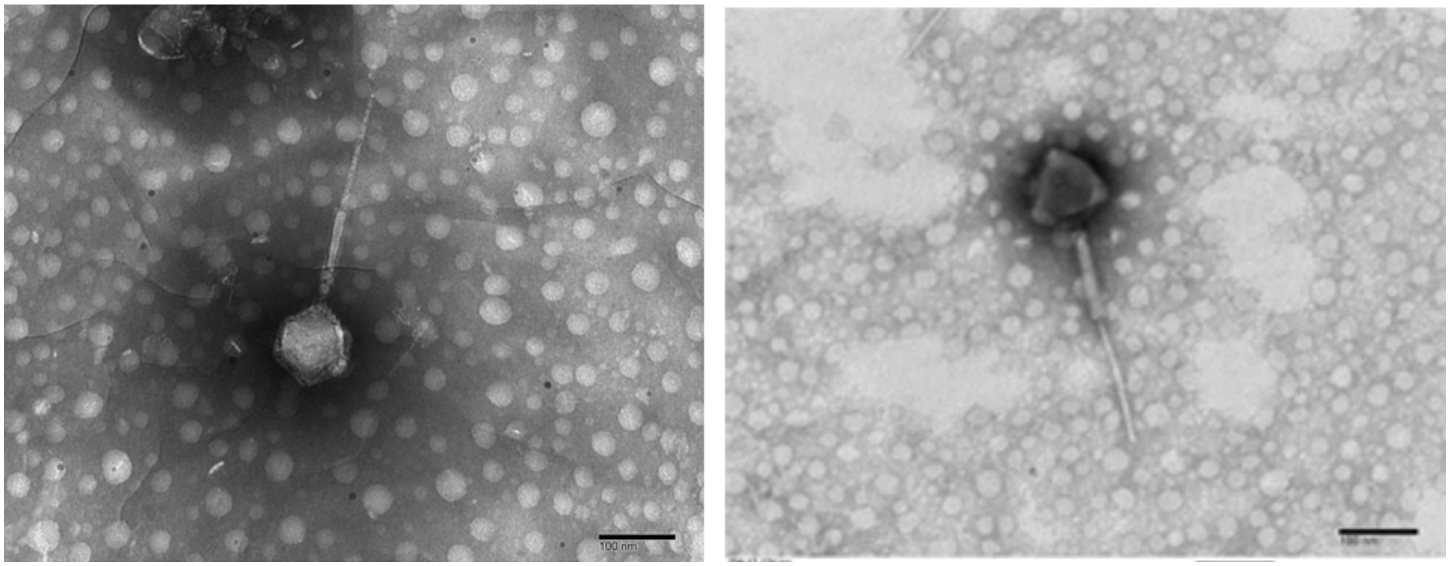

Figure 4. *Transmission Electron Micrograph of bacteriophage KP-2 capable of infecting different genotypes of ESBL-producing K. pneumonia.*

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