

Evaluation of extended-spectrum beta-lactamase production in *Escherichia coli* clinical isolates from three hospitals in Luzon, Philippines

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The increased spread of pathogens producing extended-spectrum beta-lactamase (ESBL) enzyme among members of family *Enterobacteriaceae* has been a serious problem worldwide. The absence of a fast and accurate detection protocol for ESBL producers had contributed to treatment failures in ESBL-associated infections. This study focused on the isolation of phenotypically ESBL-producing *E. coli* isolates from 3 hospitals in Luzon, Philippines. The availability of confirmed ESBLs is valuable in conducting experiments to gain information about ESBLs that are relevant to the identification of detection markers for these pathogens. Out of 119 pathogenic *E. coli* isolates from clinical samples such as urine, wound, body aspirates and blood, 37 (31%) were identified as suspected ESBLs using a preliminary test and 27 of the 37 suspected ESBLs were confirmed as ESBL producers based on a test referred to as double-disk synergy test. The highest percentage of ESBL-producing *E. coli* was obtained from urine specimens, while the lowest was from blood samples. All the ESBL-positive isolates exhibited resistance to cefotaxime and to levofloxacin. Co-resistance to other antibiotics like trimethoprim-sulfamethoxazole, ciprofloxacin, tetracycline and cefepime was also noted in some isolates. Susceptibility of ESBL-producers remains high towards carbapenems. These findings point to the urgent need to develop a fast and accurate detection protocol (including a molecular approach) for ESBL-producing pathogens.

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INTRODUCTION

Antibiotic resistance in *Escherichia coli* (*E. coli*) and other members of family *Enterobacteriaceae* is mainly due to production of the beta-lactamase enzyme that is encoded by the *bla* gene either in the chromosome or plasmid (Schmitt et al. 2007). This enzyme efficiently catalyses the irreversible hydrolysis of beta-lactam drugs such as penicillins and cephalosporins. To date, there are several variants of beta-lactamases that already exist. One of the most significant groups of beta-lactamases is the extended-spectrum beta-lactamases or ESBLs. These ESBLs are capable of hydrolyzing penicillins, first-, second- and third-generation cephalosporins, and monobactams (Bradford 2001). The production of ESBLs confers resistance to these antibiotics. The increase in the number of antibiotic-resistant pathogens and their rapid spread in the environment are a cause for concern. Research and public awareness on problems associated with ESBL-related infections are deemed important.

Most ESBLs are derivatives of the more common beta-lactamases known as TEM or SHV types (Paterson and Bonomo 2005). A few point mutations at selected loci within the genes of these enzymes gave rise to the "extended-spectrum" phenotype, allowing the ESBLs to have expanded hydrolytic activity against the second- and third-generation cephalosporins. Phenotypically, non-ESBLs show resistance only to penicillins and first-generation cephalosporins, while ESBLs have extended-resistance to second- and third-generation cephalosporins and monobactams. The hydrolytic activity of ESBLs, however, can

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be inhibited by a beta-lactamase inhibitor like clavulanic acid (Bradford 2001, Paterson and Bonomo 2005). The procedures performed in this study to identify ESBL-producing *E. coli* were based on those findings.

Infections caused by ESBL-producing pathogens are more difficult to treat compared to non-ESBL producers due to the very limited antibiotic options available. The increasing prevalence rate of ESBL-caused infections has also increased the mortality rate among affected patients (Melzer and Petersen 2007). In addition, dissemination of ESBL-producing strains is becoming more prevalent due to the inability to detect the presence of ESBLs in routine laboratory susceptibility testing methods (Subha and Ananthan 2002). Many clinical laboratories do not exert much effort in detecting ESBL production by Gram-negative bacteria because the procedure is considered by many investigators as too complex and costly. Undetected ESBL production among clinical isolates often results in inappropriate treatment with antibiotic drugs and treatment failure (Al-Jasser 2006).

Escherichia coli and *Klebsiella pneumoniae* have been the most commonly identified organisms producing ESBL. Antimicrobial surveillance data on the prevalence of extended-spectrum beta-lactamase producing isolates in the Asia-Pacific region in 1998-1999 identified the Philippines as one of the countries with high prevalence of ESBL-producing *K. pneumoniae* (Bell et al. 2002). Antimicrobial resistance monitoring data in 2002-2010 revealed that 28.2% of *E. coli* and 22.1% of *K. pneumoniae* isolates in the Asia-Pacific region are ESBL-producers, with China and Thailand having the highest proportions (Huang et al. 2012). In the Philippines, antimicrobial surveillance data in the early part of 2001 showed that *E. coli* generally have low resistance rates (1-10%) to third generation cephalosporins (Carlos 2002). In 2003, however, increase in resistance of Gram-negative bacilli to ceftazidime, ceftriaxone and cefotaxime, was reported at the Makati Medical Center with 20.1% ESBL prevalence among *E. coli* and *Klebsiella* spp. (Villanueva et al. 2003), and the Philippine General Hospital with 29.9% prevalence of ESBL-production among *Enterobacteriaceae* (Bomasang and Mendoza 2003). At the Mindanao Sanitarium and Hospital in Southern Philippines, an increase in antibiotic resistance among members of *Enterobacteriaceae* was observed from 1.69% in 2005 to 7.38 % in 2008 (Lucena et al. 2012).

This study is focused on the evaluation of ESBL production among *E. coli* clinical isolates from three selected hospitals in Luzon, Philippines. Experiments were conducted to determine the prevalence of ESBLs from different types of clinical isolates. Moreover, this research was conducted in order to obtain *E. coli* isolates that exhibit a phenotype observed for ESBL producers. Availability of ESBL-producing *E. coli* will allow the conduct of further studies relevant to the development of molecular detection procedures for ESBL-producing *E. coli*. Studies on ESBL-producing *E. coli* will contribute to improved clinical therapy and management of infections, and reduced treatment failure and mortality rates in patients affected with ESBL-positive pathogens.

MATERIALS AND METHODS

Collection of clinical isolates

Escherichia coli clinical isolates from different specimens such as blood, urine, and body fluids were grown in MacConkey selective media and maintained in nutrient agar. Collection of bacteria was conducted for a period of one year (May 2012 - May 2013) by three collaborating hospitals: the Angeles University Foundation Medical Center (Angeles City), Jose B. Lingad Memorial General Hospital (Pampanga), and Armed Forces of the Philippines Medical Center (Quezon City) in Luzon, Philippines. The isolates were identified in the hospital laboratories using conventional biochemical tests, API 20E (bioMérieux, USA) and automated bacterial ID system (VITEK 2 Systems (bioMérieux, USA).

Confirmatory Test and Determination of Prevalence of ESBL-Producing *E. coli*

Collected isolates that were reported resistant to any of the third generation cephalosporins (cefotaxime, ceftazidime, cefpodoxime, ceftriaxone) or monobactam (aztreonam) by the routine laboratory susceptibility test were classified as “suspected ESBL-producers” and were subjected to phenotypic confirmatory testing.

Phenotypic confirmatory test was performed using the double-disk synergy test or DDST (Jarlier et al. 1988, Cabrera 2004). This employs the Kirby-Bauer disk diffusion technique on a Mueller Hinton agar plate. Disks containing oxyimino-beta-lactams, like cefotaxime (30 mg) and ceftazidime (30 mg) were placed 20 - 30 mm apart (center to center) from a disk containing amoxicillin/clavulanate (20 mg/10 mg). Enhancement or extension at the edge of the zone of inhibition between the disc with cefotaxime (or ceftazidime) and the disk containing beta-lactamase inhibitor (amoxicillin/clavulanate) indicates production of ESBL by the bacterium (Figure 1A). Absence of such zone confirms non-ESBL production by the isolate (Figure 1B).

The percentage prevalence of phenotypically confirmed ESBL-producing *E. coli* from the clinical samples used in this study was determined.

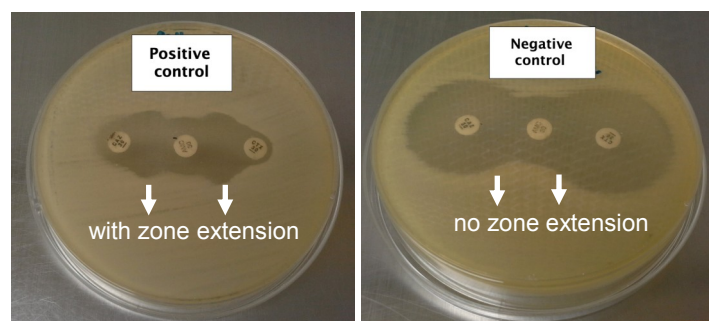


Figure 1. Positive and negative results in a double-disk synergy test (DDST). A, ESBL-production in the positive control *K. pneumoniae* 700603 with the formation of zone extension between a third generation cephalosporin (ceftazidime, CAZ or cefotaxime, CTX) and a disk containing a beta-lactamase inhibitor (center disc); B, Absence of zone extension in the non-ESBL producing negative control *E. coli* 25922.

Distribution of Confirmed ESBL-producing *E. coli* in Clinical Sources

The percentage distribution of phenotypically confirmed ESBL-producing *E. coli* in clinical samples from blood, urine, wound, aspirate and sputum was determined and analyzed.

Susceptibility of ESBL-producing *E. coli* to Other Antibiotic Agents

The susceptibility pattern to major classes of antibiotics (such as fluoroquinolone, tetracycline, folate-pathway inhibitors, aminoglycosides, beta-lactam and beta-lactamase inhibitors) of the putative ESBL-producing isolates was tested and analyzed, following the interpretive criteria for *Enterobacteriaceae* in Table 2A of the Clinical Laboratory Standards Institute M100-S23 Guideline (CLSI 2013).

RESULTS AND DISCUSSION

Collection of clinical isolates

A total of 119 non-repetitive clinical isolates identified as *E. coli* and randomly collected from blood (14), aspirate (13), sputum (7), wound (16) and urine (69), were kindly provided by the three collaborating hospitals: Angeles University Foundation Medical Center, Jose B. Lingad Memorial General Hospital and Armed Forces of the Philippines Medical Center. The isolates were identified on the basis of their characteristic morphology in selective media. Isolates were generally observed as dry, pink colonies on MacConkey media. Biochemical tests showed the ability of isolates to ferment carbohydrates (including lactose) and the ability to produce indole and lysine decarboxylase enzyme.

Confirmatory test for ESBL production

Thirty seven (37) of the 119 clinical isolates collected were reported to exhibit resistance to at least one of the third-generation cephalosporins (cefotaxime, ceftazidime, cefpodoxime, ceftriaxone) or monobactam (aztreonam). These thirty seven isolates were considered "suspected ESBL-producers". The confirmatory test (referred to as the double-disc synergy test or DDST) on the 37 "suspected ESBL-producing" *E. coli* isolates suggested that 27 out of the 37 were ESBL-producers. The 27 confirmed ESBL-producing isolates (Figure 2) were observed to exhibit hydrolytic activity towards the third-generation cephalosporin (cefotaxime or ceftazidime) used. This hydrolytic activity was observed by their resistance toward the antibiotic (ability to grow around the antibiotic disk). In the presence of a beta-lactamase inhibitor like clavulanic acid in amoxicillin/clavulanate, however, the hydrolytic activity of ESBL was inhibited (consistent with the expected result for ESBL producers). Inhibition by a beta-lactamase inhibitor was observed as an enhancement of the zone of inhibition between the disk with third-generation cephalosporin (cefotaxime or ceftazidime) and the disk containing the beta-lactamase inhibitor, clavulanic acid (AMC). Isolates that do not produce ESBL cannot inactivate the antibiotics used and are classified as non-ESBL-producers, indicated by the absence of enhancement or extension at the edge of the zone of inhibition (Figure 2B).

The 27 ESBL-producing isolates all showed marked resis-

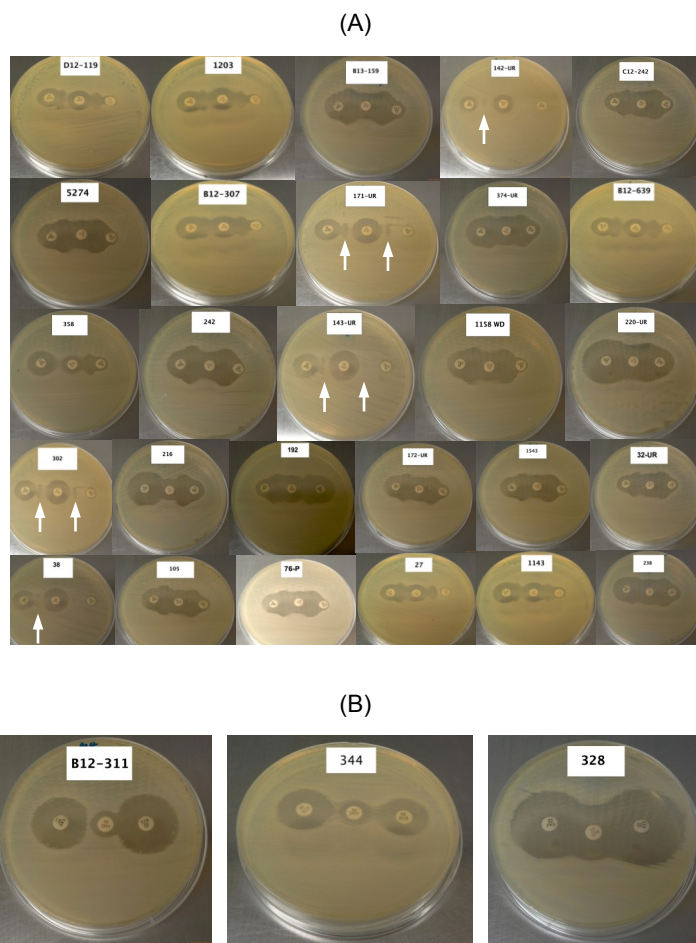


Figure 2. Results of the confirmatory double-disc synergy test (DDST) for ESBL production. A, the 27 *E. coli* isolates that were confirmed as ESBL-producers showing the characteristic enhancement or extension in the zone of inhibition between the cephalosporin disk (ceftazidime, CAZ, or cefotaxime, CTX) and a beta-lactamase inhibitor (clavulanic acid in amoxicillin/clavulanate, AMC). Zone enhancement for isolates labeled 142-UR, 171-UR, 143-UR, 302 and 38 was not as prominent as with the rest (indicated by arrows), which may improve with smaller distance between the disks. B, examples of phenotypically non-ESBL producers with absence of enhancement or extension in the zone of inhibition between the CAZ (or CTX) and AMC disk.

tance to cefotaxime (CTX), resulting in a very small zone of inhibition around the CTX disk, as shown by representative samples in Figure 3A. This small inhibition zone was augmented by the beta-lactamase inhibitor disk (AMC) placed next to it. Isolates 358, 220-UR and 216 were initially reported to be resistant to at least one of the third-generation cephalosporins including cefotaxime (CTX). Isolates 220-UR and 216 were both susceptible to ceftazidime (CAZ), while isolate 358 has intermediate susceptibility to CAZ in the routine antibiotic susceptibility testing. Confirmatory test by DDST revealed that all these three isolates are ESBL-producers, due to their ability to hydrolyze the third-generation cephalosporin (cefotaxime or ceftazidime), and the ability of the beta-lactamase inhibitor (clavulanic acid) to inhibit the activity of the beta-lactamase produced by the bacteria, as indicated by the extension at the edge of the zone of inhibition.

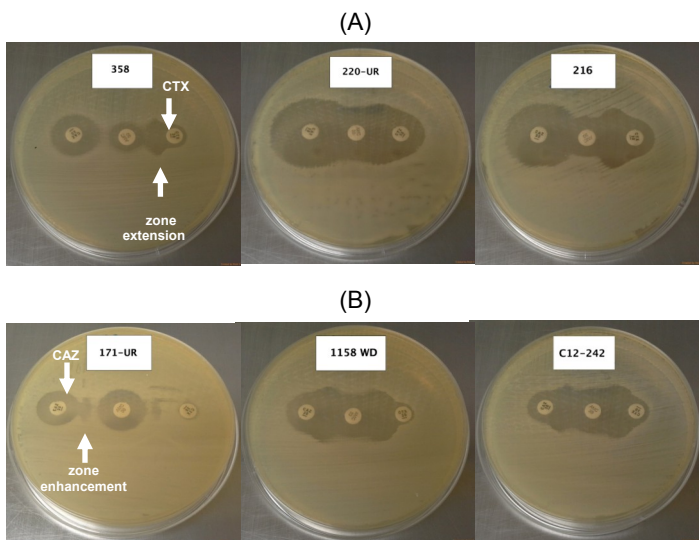


Figure 3. Examples of isolates with marked resistance to cefotaxime (A) and to both cefotaxime and ceftazidime (B). Resistance, which is indicated by the small zone around the cephalosporin disk (CAZ or CTX), has been inhibited by the beta-lactamase inhibitor (AMC), as observed by the extension or enhancement of the zone of inhibition between the cephalosporin disk (CAZ or CTX) and a beta-lactamase inhibitor (AMC).

Twenty out of twenty seven are also resistant to ceftazidime, with zones of inhibition that were also enhanced by the AMC disk as shown by some representative samples in Figure 3B. Isolates labeled 171-UR, 1158 and C12-242 were all reported to be resistant to cefotaxime and ceftazidime in the routine antibiotic susceptibility testing, and were also confirmed as ESBL-producers by DDST.

Distribution of phenotypically confirmed ESBL-producing *E. coli* in clinical sources

The highest percentage distribution of ESBL-producing *E. coli* was noted in urine samples (62%), followed by wound (19%) specimens (Figure 4). It should be noted, however, that most of the clinical isolates were from urine (58%). *Escherichia coli* is almost equally obtained from other body sites like wound, blood and body fluids or aspirate. The possible occurrence of ESBL-producing *E. coli* in sterile critical sites such as in blood (4%) was also observed.

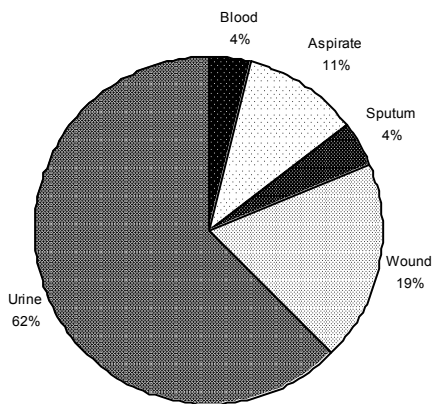


Figure 4. Distribution of confirmed ESBL-producing *E. coli* in clinical sources.

Susceptibility pattern of ESBL-producing *E. coli*

The antibiotic susceptibility results for all the 27 phenotypically confirmed ESBL-positive isolates showed 100% resistance to cefotaxime and levofloxacin (Figure 5). Co-resistance to other antibiotics such as trimethoprim-sulfamethoxazole (93%), ciprofloxacin (88%), tetracycline (75%), amoxicillin-clavulanic acid (73%) and ticarcillin-clavulanic acid (93%) was also observed. Susceptibility rates of all the identified ESBL-positive *E. coli* to amikacin, imipenem, meropenem and ertapenem were high.

This study is focused on obtaining putative and confirmed ESBL-producing *E. coli* from 119 clinical bacterial isolates collected from three hospitals in Luzon, Philippines. The isolation of putative and confirmed ESBL-producing *E. coli* after conducting a preliminary conventional antibiotic susceptibility assay (Kirby Bauer disk diffusion method) and a confirmatory test called double-disk synergy test (DDST), will pave the way for conducting further studies, including molecular approaches, on bacteria with such phenotypes.

Interest in studying ESBL-producing *E. coli* is heightened because these bacteria have been implicated in various types of infection that remain to be a challenge due to the difficulty in therapeutic management (Rawat and Nair 2010). Early detection of ESBL-producing pathogens will significantly help clinicians in the selection of appropriate antibiotics for better therapeutic regimens. A molecular approach that involves detecting gene sequences implicated in ESBL production is expected to be fast and accurate. In order to develop a molecular detection procedure, detailed studies on gene sequences from suspected and confirmed ESBL-producing *E. coli* are necessary. Before this could be done, non-ESBL control *E. coli* isolates and the isolates exhibiting an ESBL-positive phenotype need to be isolated and evaluated.

In this study, 37 of the 119 *E. coli* isolates (31%) were suspected as ESBL-producers based on antibiotic resistance to one of the several third-generation cephalosporins used. The use of more than one antibiotic for screening improves the detection of ESBL-producers because their hydrolytic activity towards a given antibiotic may vary as a result of inoculum effect and substrate specificity (Rawat and Nair 2010). Out of 37, only 27 were positive in the ESBL confirmatory tests (DDST) showing that out of 119 total isolates, 23% were phenotypically confirmed ESBL-producers. This value is comparable to the prevalence of ESBLs reported in 2002 to 2010 at 28.2% for *E. coli* and 22.1% for *K. pneumoniae* in the Asia-Pacific region (Huang et al. 2012). The 23% prevalence of ESBLs in 119 isolates in this study from three hospitals in Luzon, Philippines appears to be higher compared to that in previous surveys in the country, with 20.1% ESBLs from combined *E. coli* and *Klebsiella* spp. isolates reported for the Makati Medical Center (Villanueva et al. 2003) and 29.9% prevalence of ESBL-production among isolates belonging to *Enterobacteriaceae* reported for the Philippine General Hospital in 2003 (Bomasang and Mendoza 2003).

The double-disk synergy test is the most widely used test to phenotypically confirm ESBL-production by a pathogen (Ho et al. 2000) and is considered a reliable method for the detection of

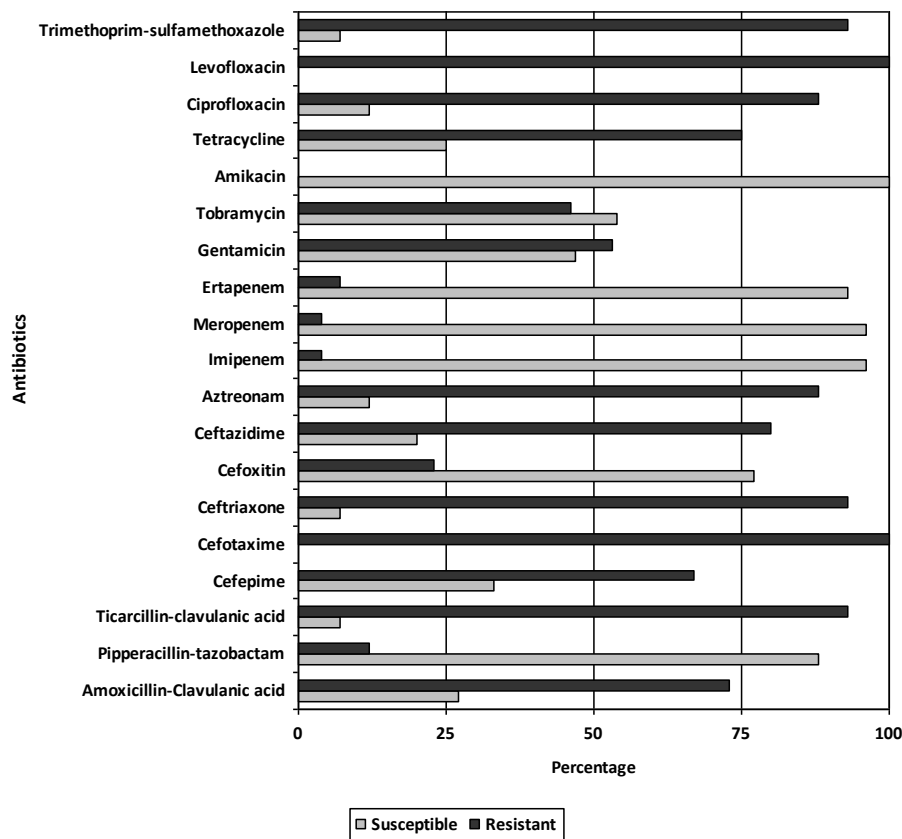


Figure 5. Antibiotic susceptibility pattern of ESBL-producing *E. coli*.

ESBLs (Bradford 2001). Extended-spectrum beta-lactamase producers can inactivate even the broad-spectrum, second- and third-generation cephalosporins. This is due to mutations in the active site of the enzyme that allow better hydrolysis of the beta-lactam substrates which, at the same time, make them susceptible to beta-lactamase inhibitors like clavulanic acid. Thus, during screening by DDST, ESBL-production is determined by the reduction in the zone of inhibition around the antibiotic disk containing a third-generation cephalosporin, and an enhancement or extension at the edge of this zone between the cephalosporin disk and the disk containing a beta-lactamase inhibitor (Figure 1).

Analysis of the prevalence of ESBL-positive isolates from the different specimen sources revealed that 62% ESBL isolates were from urine samples (Figure 4). Pathogenic *E. coli* is a very common pathogen causing community-acquired urinary tract infection (Alipourfard and Nili 2010, Lucena et al. 2012). The uropathogenic *E. coli* strains are the most commonly exposed to antibiotic treatment, often as a result of recurring infection. Nineteen percent of the ESBL-isolates were obtained from wound samples probably because patients with infected wounds also take antibiotics even without doctor's recommendation, resulting in the development of strains with increased resistance to antibiotics. The lower rate of ESBL-associated infections in blood may reflect the ability of ESBL-producing *E. coli* to cause a life-threatening infection like bacteremia (presence of bacteria in blood), or, worse, sepsis, a condition characterized as a severe blood infection that leads to organ failure and death (Rawat and Nair 2010).

Susceptibility evaluation of isolates to several antibiotics revealed that all 27 confirmed ESBLs were resistant to cefotaxime and to levofloxacin, an antibiotic that belongs to another group called fluoroquinolones (Figure 5). Among the third generation cephalosporins, cefotaxime is one of the recommended first-line treatments for *E. coli*-caused infections. This may explain the observed resistance of all ESBL-positive isolates to cefotaxime. The improper or over use of this antibiotic may have resulted in developing resistance to the drug. Ceftazidime, also classified as a third-generation cephalosporin, is not routinely used and the rate of resistance to this antibiotic is lower at 80% compared to cefotaxime.

Co-resistance of ESBLs was also observed to the following groups of antibiotics: folate pathway inhibitors (e.g., trimethoprim-sulfamethoxazole), fluoroquinolones (e.g., ciprofloxacin), tetracycline, and beta-lactam/beta-lactamase inhibitor combinations (e.g., amoxicillin-clavulanic acid and ticarcillin-clavulanic acid). A marked decrease in the susceptibility to cefepime (33%) from the reported rate of 96% in the past decade by Villanueva et al. (2003) was also noted. This could be interpreted as an extended activity to fourth-generation cephalosporin. So far, the only antibiotics that can effectively be used for ESBL-associated infections are the carbapenems: imipenem, meropenem and ertapenem. Amikacin (an aminoglycoside) and piperacillin-tazobactam (beta-lactam/beta-lactamase inhibitor combination) also showed good activity against ESBL-producing *E. coli*. The use of an aminoglycoside alone, however, is not recommended for serious infections, and is better used in conjunction with other drugs for efficient bactericidal activity (Paterson 2007).

Based on the results of the susceptibility evaluation, the treatment of infection due to ESBL-producers would require the use of a highly potent group of antibiotics because bacterial resistance to several of antibiotics was observed in ESBL producers. Without the detection of ESBLs, proper treatment could not be administered, because routine treatments do not involve a highly potent group of antibiotics. An accurate confirmation of the presence of ESBL pathogens is obviously very important for the administration of appropriate antibiotics.

The problem of ESBL-associated infections can therefore cause serious medical and economic impact to every country including the Philippines. Antimicrobial therapy of infections due to ESBL-producing pathogens is a big challenge to the clinical practitioners. It is therefore advantageous if hospital surveillance of ESBLs is an integral component of infection control, in order to strictly monitor emerging multi-drug resistant pathogens. Identification of molecular markers to detect ESBLs in the shortest time possible will be a significant contribution to infection treatment and management. The isolation of several strains of ESBL-producing *E. coli* that we did in this study is an initial and crucial step in elucidating the molecular basis for differentiating ESBL-producing from non-ESBL phenotypes. A follow-up elucidation of ESBL-specific gene sequences will be useful in developing an accurate and fast detection protocol for ESBLs.

CONCLUSION

The prevalence of ESBL-producing *E. coli* among the three selected hospitals shows that resistance due to ESBL continues to rise and remains a clinical problem posing a threat in the field of antibiotic therapy. The isolation of ESBLs from different samples indicates how this enzyme can complicate antibiotic treatment of common infections. The presence of co-resistance to other antibiotics can further lead to more serious infections due to limited antibiotic options. It is therefore imperative that research, which could contribute to the development of early and accurate detection of ESBL-producing pathogens, be done in order to reduce treatment failures and prevent further development and spread of antibiotic resistance.

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CONFLICTS OF INTEREST

There is no conflict of interest among authors, institutions and individuals mentioned above in the conduct of this study and the preparation and submission of this manuscript.

CONTRIBUTION OF INDIVIDUAL AUTHORS

Mrs. Catherine Bacani and Mrs. Alma Mendoza assisted Ms. Merlyn Cruz in conducting the experiments in this study. Ms. Cruz conceptualized the study, analyzed the data and prepared the manuscript. Dr. Cynthia Hedreyda contributed to data analysis and preparation of the manuscript.

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