Detection of Plasmid-Borne β-Lactamase Genes in Extended-Spectrum β-Lactamase (ESBL) and Non-ESBL-Producing Escherichia coli Clinical Isolates

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Increasing frequency of infections caused by antibiotic resistant Escherichia coli strains producing extended-spectrum β-lactamase (ESBL) needs to be addressed by continuous surveillance and accurate detection of specific ESBLs genes for more effective treatment. A total of 71 β-lactam drug resistant isolates (26 phenotypically ESBL-producing and 45 non-ESBL-producing) were observed to carry approximately 23 kb plasmids. These isolates were subjected to β-lactamase gene-targeted PCR to detect plasmid-encoded blaTEM, blaSHV, blaCTX-M group1 and blaCTX-M group9 genes. BLAST analysis of amplicons revealed that plasmid-encoded blaTEM is most prevalent in both ESBL and non-ESBL-producing E. coli isolates. Plasmid-encoded blaSHV gene was only detected in 8 non-ESBL-producing isolates and explanation of such observation awaits additional studies to detect the possibility that the gene could be in the chromosomal DNA or to test the prevalence of the plasmid-encoded gene with more isolates. Twelve isolates of the ESBL-type blaCTX-M were identified from phenotypically identified ESBLs, comparable with 13 isolates detected with blaTEM. This observation suggests that the relatively newly emerging ESBL-type CTX-M is continuously increasing as one of the new β-lactamase derivatives among ESBL-producing E. coli in the clinical setting. This study reveals that there is discrepancy between the results of the phenotypic observation and genotypic analysis showing that the presence of ESBL-associated β-lactamase genes may be undetected when using the conventional phenotypic approach. Mutation in these unexpressed genes may result to ESBL antibiotic resistance, suggesting that the unexpressed and undetected genes may serve as reservoir for ESBL genes.

Key words: CTX-M, ESBL, Escherichia coli, SHV, TEM

INTRODUCTION

Drug resistance among bacteria is largely attributed to their production of β-lactamase enzyme that can hydrolyze or inactivate the β-lactam drugs that interfere with bacterial cell wall synthesis (Bauman 2004). β-lactam drugs include the antibiotics penicillins, cephalosporins, cephamycins, carbapenems, mono-bactams and β-lactamase inhibitors. Infections caused by members of Enterobacteriaceae such as Escherichia coli, are most often difficult to treat due to their resistance particularly to β-lactam drugs.

Innovations in drug technology introduced new derivatives of β-lactam drugs that are more potent, broader in spectrum of target organisms and more resistant to the hydrolytic activity of β-lactamase enzyme. When a new
β-lactam drug derivative is developed, new β-lactamase derivatives usually emerge, as a result of the selective pressure due to the use and abuse of the β-lactam drug. Because of the increased spectrum of bacterial activity against new expanded-spectrum β-lactams, particularly the cephalosporins, the β-lactamases are called extended-spectrum β-lactamases or ESBLs (Paterson and Bonomo 2005). In general, ESBLs are β-lactamases that are capable of conferring resistance to penicillins, first-, second- and third-generation cephalosporins, by hydrolyzing these antibiotics. ESBLs, however, are inhibited by β-lactamase inhibitors such as clavulanic acid (Paterson and Bonomo 2005).

The rise in number of antibiotic resistant pathogens were shown in some antimicrobial surveillance data conducted in the Philippines. The prevalence of ESBL-producing Enterobacteriaceae was reported at the Makati Medical Center (Villanueva et al. 2003) and the Philippine General Hospital (Bomasang and Mendoza 2003). A similar observation was reported by Lucena et al. (2012) at Mindanao Sanitarium and Hospital in Southern Philippines, which showed an increase in resistance of Gram-negative from 1.69% in 2005 to 7.38% in 2008. In a retrospective study on ESBL-producing bacteria in the Philippines from 1999-2013, the prevalence of ESBL-producing K. pneumoniae and E. coli ranged from 10-43.24% and 4-20.9%, respectively (Lota and Latorre 2014).

Different groups of the β-lactamase gene (bla) are found either in chromosomal DNA or plasmid (Schmitt et al. 2007). The presence of the gene in plasmids further facilitated its transfer to different species of bacteria. The TEM and SHV β-lactamases were the first plasmid-mediated enzymes, from which many of the ESBLs have been derived (Bush and Jacoby 2010). A few point mutations at selected loci within the gene encoding these enzymes gave rise to the extended-spectrum phenotype, which now accounts for many ESBLs identified. TEM and SHV-derived ESBLs can hydrolyze the third generation cephalosporins particularly ceftazidime. At the beginning of the 21st century, a new family of plasmid-mediated ESBLs called CTX-M, which preferentially hydrolyze cefotaxime, became predominant in European countries, and started to spread in Southeast Asia including the Philippines.

Monitoring of ESBLs should not be limited to phenotypic screening since there had been reports showing the discrepancy in the phenotypic and genotypic detection (Yazdi et al. 2012; Xu et al. 2014). This study is focused on the isolation of plasmids from ESBL-producing and non-ESBL-producing E. coli clinical isolates and PCR detection of plasmid-borne β-lactamase genes (bla TEM, bla SHV, bla_CTX-M group1 and bla CTX-M group9) that confer antibiotic resistance phenotype. The relative distribution of each gene among the E. coli isolates studied was analyzed and presence of unexpressed ESBL-associated genes in phenotypically non-ESBL isolates was evaluated. The presence of unexpressed ESBL genes may confer ESBL phenotype to pathogens after acquiring the needed mutation. It is therefore necessary to consider unexpressed ESBL genes in making appropriate steps to address problems associated with the increasing frequency and rapid spread of ESBL-producing bacteria. Consequently, the new information can be useful in developing accurate protocols for detecting ESBLs and treating infections.

**METHODS**

*Escherichia coli* Clinical Isolates

A total of 72 β-lactam resistant clinical E. coli isolates from selected hospitals in Luzon, Philippines (Cruz et al. 2014) were subjected to plasmid isolation procedure. Seventy-one out of 72 isolates that were positive for plasmid included 45 isolates classified as phenotypically non-ESBL and 26 isolates classified under phenotypically ESBL based on results of a previous study (Cruz et al. 2014) using the Double-disk Synergy Test (DDST).

Plasmid Extraction

*Escherichia coli* isolates were sub-cultured in Luria-Bertani broth and incubated for 24 hours prior to extraction. Plasmid was extracted from each isolate using commercially available plasmid extraction kit (Purelink Quick Plasmid MiniPrep by Invitrogen, USA). Extracted plasmids were subjected to agarose (0.8%) gel electrophoresis and visualized under UV (Alpha Innotech, USA) after staining with ethidium bromide and photographed by Alpha DigiDoc Pro (Alpha Innotech, USA).

Amplification of β-Lactamases Genes (bla)

Primers designed for detecting TEM-, SHV- and CTX-M (groups 1 and 9) β-lactamase-encoding genes (Dallenne et al. 2010, Figure 1), plasmid DNA templates from E. coli clinical isolates and available positive (TEM-1-producing E. coli ATCC 35218; CTX-M and SHV-producing clinical isolates confirmed by sequencing) and negative control (non-β-lactamase-producing E. coli ATCC 25922) isolates, were used in PCR. PCR was performed in a total volume of 25 µL PCR reaction containing 0.4 µM of each of pair of primers (Invitrogen, USA), 1 µL of DNA template, 0.4 mM dNTPs, 1.0-1.25 mM MgCl2, 1 U of Pfx polymerase (Invitrogen, USA) and 5 uL of 10X amplification buffer.

The conditions for PCR included initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 15 sec, annealing at 59, 57, 61, and 58°C (for primers
targeting \( \texttt{bla}_{\text{TEM}} \), \( \texttt{bla}_{\text{SHV}} \), \( \texttt{bla}_{\text{CTX-M group1}} \), and \( \texttt{bla}_{\text{CTX-M group9}} \), respectively) for 30 sec and extension at 68°C for 1 min, and final extension at 68°C for 4 min. Amplicons were visualized and photographed after agarose (1%) gel electrophoresis and ethidium bromide staining using Alpha DigiDoc Pro (Alpha Innotech, USA).

Identification and Distribution of \( \texttt{bla} \) Genes

Amplicons were sent for sequencing to 1st BASE (BASE Life Sciences Holdings, Singapore). Sequence data were compared to reference sequences available in the database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) sequence alignment software to identify the specific \( \texttt{bla} \) gene detected. Presence of one or more \( \texttt{bla} \) genes in each isolate was evaluated.

RESULTS

Plasmid-Borne \( \beta \)-Lactamase Genes (\( \texttt{bla} \))

Plasmid profiles revealed that 71 isolates have one or more plasmids with bands representing amplicons ranging from 2 to 23 kb. A large band that is approximately 23 kb, was found common to all 71 isolates (Figure 2). Table 1 presents the number of ESBL and non-ESBL isolates that elicited expected amplicons after \( \beta \)-lactamase gene-targeted PCR using extracted plasmids from each isolate as template and primers that target four types of \( \beta \)-lactamase genes including the plasmid-mediated \( \beta \)-lactamase TEM-1, plasmid-encoded SHV-1, and two groups under CTX-M (CTX-M group 1 and CTX-M group 9; Bush and Jacoby 2010). Amplicons of about 800 bp, 688 bp and 561 bp that were expected for amplified

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**Figure 1.** Annealing sites for primer sets targeting the major types of \( \beta \)-lactamase gene. The expected sizes of amplified internal gene fragments are 800 bp for \( \texttt{bla}_{\text{TEM}} \), 713 bp for \( \texttt{bla}_{\text{SHV}} \), 688 bp for \( \texttt{bla}_{\text{CTX-M group1}} \), and 561 bp for \( \texttt{bla}_{\text{CTX-M group9}} \).
β-lactamase genes for TEM ($bla_{\text{TEM}}$), CTX-M group 1 ($bla_{\text{CTX-M\_group1}}$), and CTX-M group 9 ($bla_{\text{CTX-M\_group9}}$), respectively, were observed (Figures 3 and 4).

Detection of β-Lactamase Genes ($bla$)

β-lactamase gene-targeted PCR primers (Figure 1) were used to amplify and detect $bla$ which encode the four types of β-lactamase under Class A (Bush and Jacoby 2010), including the plasmid-mediated β-lactamase TEM-1, plasmid-encoded SHV-1, and two groups under CTX-M (CTX-M group 1 and CTX-M group 9). The number of ESBL-producing and non ESBL-producing isolates that exhibited expected amplification products of PCR using each type of β-lactamase gene-targeted primers are summarized in Table 1.

Table 1. Results of β-lactamase gene-targeted PCR detection and identification.

<table>
<thead>
<tr>
<th>$bla$ gene detected</th>
<th>Number of isolates positive for expected size amplicon</th>
<th>Number of isolates positive for gene based on sequence analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ESBL</td>
<td>Non-ESBL</td>
</tr>
<tr>
<td>TEM (~800 bp)</td>
<td>13</td>
<td>27</td>
</tr>
<tr>
<td>SHV (~713 bp)</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>CTX-M group 1 (~688 bp)</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>CTX-M group 9 (~561 bp)</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>No $bla$ detected</td>
<td>7</td>
<td>17</td>
</tr>
</tbody>
</table>

Figure 3. Representative amplicons of PCR using primers that target β-lactamase genes and plasmid DNA template from phenotypically ESBL-producing $E.\ coli$ isolates. (A) Expected size amplicons (~800bp) of PCR using primers that target $bla_{\text{TEM}}$ gene are in lanes 3-6, 8 and 11. Lanes 12 and 13 are results of PCR using templates from $bla_{\text{TEM}}$-positive control (ATCC 35218) and a negative control, respectively. (B) Expected size amplicons (~688bp) of PCR using primers that target $bla_{\text{CTX-M\_group1}}$ genes are in lanes 1,6,9,11 and 14. Lane 15 is the negative control. (C) Expected size amplicons (~561bp) of PCR using primers for $bla_{\text{CTX-M\_group9}}$ genes are in lanes 1-4, 6 and 7. Lane 15 is the negative control. M is the 100-bp ladder in A and B, and 100 bp Plus DNA ladder in C. Yellow arrows point to positions of expected size of β-lactamase gene amplicons.
amplicons of about 800 bp, 688 bp and 561 bp that were expected for amplified β-lactamase genes for TEM (bla TEM ), CTX-M group 1 (bla CTX-M-1 group ), and CTX-M group 9 (bla CTX-M-9 group ), respectively, were observed in both phenotypically ESBL-producing and non-ESBL-producing isolates (Figures 3 and 4). Expected 713-bp amplicon for SHV β-lactamase gene (bla SHV ) was not produced in ESBL-producing isolates but were observed in eight non-ESBL-producers (Figure 4).

**BLAST Analysis of bla Gene Amplicons**

BLAST analysis revealed that the target gene was detected in all bla TEM -gene-targeted PCR for both ESBL and non-

ESBL-producing isolates (Table 1). Out of 13 amplicons from bla TEM -gene-targeted PCR of ESBL-producing isolates, 7 exhibited 100% while 6 exhibited 99% sequence similarity with TEM-1 type (bla TEM-1 ) gene. All the 27 amplicons of the bla TEM -gene-targeted PCR in non-ESBL-producing isolates were also positive for bla TEM gene with 17 of 27 exhibiting 100% and 10 of 27 exhibiting 99% sequence similarity with reported bla TEM-1 gene.

The bla SHV -gene-targeted PCR using plasmid DNA templates from ESBL-producing isolates did not result in the amplification of expected 713 bp product (Table 1). Expected target gene was only amplified in bla SHV -gene-targeted PCR in 8 non-ESBL-producing isolates. BLAST results showed that three of these eight isolates exhibit 100% sequence similarity of amplicons with bla SHV-1 gene, and the remaining five of eight, showed 96-99% similarity with SHV gene previously reported in ESBL-producing isolates. Nine of 10 amplicons from PCR using primers that target bla CTX-M-1 gene in ESBL-producing isolates exhibited about 99% sequence similarity with bla CTX-M-15 gene that encodes β-lactamase under CTX-M group 1. One of ten amplicons exhibited 99% sequence similarity with bla CTX-M-5, another member of CTX-M group 1. Two of the ESBL-producing isolates contain bla CTX-M gene with 99% sequence similarity with bla CTX-M-14 and bla CTX-M-27 both under CTX-M group 9. Among the non-ESBLs, one isolate was identified to carry bla CTX-M group 17.

**Distribution of bla Genes Among ESBL and Non-ESBL-Producers**

Both ESBL and non-ESBL-producing E. coli isolates exhibited highest number of isolates (13 and 27, respectively) with TEM type bla gene (Table 1). SHV gene was only detected from non-ESBL producing isolates and together with TEM. The CTX-M Group 1 was observed in both ESBL and non-ESBL producers but more isolates in ESBLs. CTX-M Group 1 gene was also detected together with TEM in five ESBL-producing isolates. Type CTX-M Group 9 was only detected from ESBL-producing isolates, one of which was detected together with TEM.

**DISCUSSION**

Antibiotic resistant ESBL and non-ESBL-producing E. coli are among the causative agents of infections that are often difficult to treat. Because ESBL-producing bacteria, in general, exhibit wider spectrum of antibiotic resistance and since increasing frequency of ESBLs is observed worldwide, infections with ESBL-producing strains experience treatment failures and are more difficult to control. As a result, the detection of ESBL-producing strains is deemed important. The widely used protocol to
detect ESBL-producing clinical E. coli isolates makes use of phenotypic evaluation using either the combination-disk diffusion assay (CLSI 2013) or the Double-disk Synergy Test (DDST, Jarlier et al. 1988). PCR detection is suggested in some reports (Dallenne et al. 2010; Ahmed et al. 2013), but more expensive and will require nucleotide sequencing to confirm results. In this study, phenotypically confirmed ESBL and non-ESBL-producing E. coli clinical isolates (based on resistance to β-lactam antibiotic, coupled with DDST) were subjected to plasmid DNA detection, bla gene detection and subsequent bla gene sequence analysis. These procedures were performed to detect the presence of four types of plasmid-borne β-lactamase (bla) gene present in each isolate. Moreover, the prevalence of four types of plasmid-borne bla genes among β-lactam-resistant E. coli, including the derivatives called ESBL, was evaluated. One main goal of the study is to determine if the routine protocol for detecting ESBL producing isolates could miss out on accurate detection of possible ESBL or presence of genes that may be potential sources of ESBL genes that may be acquired given the necessary conditions or mutations. The findings in this study highlight the significance of detecting the specific ESBL produced by the pathogen in order to monitor the emergence of ESBL derivatives, to gain information on the prevalence of specific types of bla genes in clinical isolates in the Philippines, and gain knowledge that could be useful for studies on spread of antibiotic resistance in different species. Data from the study are expected to contribute to information useful in developing more appropriate and effective strategies in laboratory detection of ESBL-producing strains.

Earlier studies reported that the β-lactamase gene is encoded either in the chromosomal DNA or plasmids (Schmitt et al. 2007). The prevalent ESBL-associated genes in E. coli, however, are reported to be plasmid-borne (Dhillon and Clark 2012). This study was therefore focused on detecting isolates with possible plasmid-encoded ESBLs from 72 E. coli clinical isolates. Only one isolate did not contain a plasmid (Figure 2.A1, lane 11) and it could be hypothesized that the bla gene that confers antibiotic resistance in this plasmid-minus isolate is in the chromosomal DNA. A plasmid of about 23 kb was consistently observed from the profiles of all 71 isolates. The 23 kb plasmids could be containing the β-lactamase genes that confer antibiotic resistance to the ESBL and non-ESBL-producing isolates. Gram negative bacteria are described to have 6 to 160 kb plasmids carrying antibiotic resistance genes (Ogbolu et al. 2013). In general, plasmid-mediated ESBLs are observed to be encoded on large conjugative plasmids and these genes have the propensity to jump between organisms that can cause outbreaks of infections, especially if these are carried by transmissible pathogens (Dhillon and Clark 2012). In this study, the 23 kb plasmids were detected from phenotypically β-lactamase producing E. coli, suggesting plasmid-mediated β-lactamase in these ESBL-producing E. coli. Conjugative dissemination of β-lactamase-carrying plasmids could be involved in facilitating the spread of antibiotic resistance among different members of the species.

To identify the type of β-lactamase genes in ESBL and non-ESBL-producing E. coli isolates, primers targeting internal fragments of plasmid-borne β-lactamase genes bla TEM, bla SHV, bla CTX-M group 1 and bla CTX-M group 9 (Dallenne et al. 2010) were used to amplify the target genes using plasmid DNA from each isolate as template. Results showed that bla TEM (800 bp amplicon) was most prevalent among the β-lactamase genes detected for both ESBL (13/26 or 50%) and non-ESBL-producing (27/45 or 60%) E. coli isolates (Table 1). TEM-1 β-lactamase enzyme was the first identified plasmid-mediated β-lactamase in Gram-negative (Medeiros 1984). No bla SHV gene (713 bp amplicon) was detected from any of the 26 phenotypically confirmed ESBL-producing E. coli isolates. The SHV β-lactamase enzyme is reported to be more often encoded in the chromosomal DNA (Bradford 2001) consistent with the observation that this gene is absent in most of the isolates studied. Another study may be focused on confirming the presence of the bla SHV gene in the chromosomal DNA. It was also observed however that the bla SHV gene was observed in 8 out of 45 non-ESBL-producing isolates (five showed 96 to 99% sequence similarity with same genes previously reported in ESBL-producers). Interpretation of the presence of the bla SHV gene in non-ESBL-producing E. coli compared to ESBLs awaits further analysis using more clinical isolates and additional sequence analysis of the genes in different isolates. Similar studies on isolates from other hospitals and other regions of the country could shed light on prevalence of bla SHV gene among non-ESBL-producing E. coli.

The CTX-M group is considered a relatively new family of plasmid-mediated β-lactamase that preferentially hydrolyze cefotaxime (Lartigue et al. 2006). Results of this study, however, revealed that 10 of 26 (38%) ESBL-producing E. coli isolates have genes encoding for this enzyme, where 5 of ten were present together with bla TEM. A total of 12 out of 26 ESBL-producing isolates carry bla CTX-M belonging to group 1 (688 bp amplicon) and group 9 (561 bp amplicon), comparable to the prevalence of bla TEM (13 of 26), suggesting a quick spread of this relatively newly detected gene among clinical isolates of E. coli. In the early part of 2000, CTX-M ESBL is believed to be the dominant type in East Asia (Komatsu et al. 2001; Bonnet 2004). The predominance of CTX-M type ESBL among clinical isolates has also been reported in the studies conducted in other hospitals in the Philippines (Tian et al. 2010; Lucena et al. 2012). Only one non-ESBL-producing isolate contains the CTX-M group 1
gene. This could be interpreted as a possible example of a strain that possesses the gene for ESBL that is, nevertheless inactive or unexpressed. Extensive sequence analysis of the complete gene from this isolate is necessary to confirm if the bacterium contains unexpressed ESBL gene, possibly with a mutation in the structural gene or its regulatory region. Sequence analysis of the complete gene could reveal the reason why the presence of the gene did not result to an extended-spectrum antibiotic resistance. The existence of inactive or unexpressed ESBL genes was reported from isolates of K. pneumoniae in China by Xu et al. (2014), indicating that “silencing” of antibiotic resistance in clinical isolates can possibly occur.

In this study, PCR using plasmids from seven ESBL-producers and 17 non-ESBL-producers (Table 1) did not result to the amplification of any of four types of \( \beta \)-lactamase genes. The PCR detection of \( \beta \)-lactamase genes in this study is limited to four pairs of primers detecting only three groups (TEM, SHV, and CTX-M) of \( \text{bla} \) genes. The \( \beta \)-lactam resistance genes in these isolates maybe in the chromosomal DNA, or could belong to other groups of \( \beta \)-lactamase like AmpC, PC-1, OXA, or CepA (Bush and Jacoby 2010) which would require different sets of primers for amplification. Inability to detect \( \beta \)-lactamase genes, if present, could also be due to sequence variation within the primer annealing sites. TEM group for example, has more than 200 \( \text{bla}_{\mathrm{TEM}} \) genotypes, SHV group has more than 190 \( \text{bla}_{\mathrm{SHV}} \) genotypes and more than 130 CTX-M variants have been described, on the basis of the amino acid sequences (http://www.lahey.org/ Studies). Nucleotide sequencing is therefore essential to analyze the entire gene sequence in order to accurately identify an ESBL derivative since the few point mutations could be the difference between an ESBL from a non-ESBL gene. Thus, the presence of ESBL-associated \( \beta \)-lactamase genes in a non-ESBL-producing \( E. \ coli \), may serve as potential reservoirs for antibiotic resistance, as they can continuously acquire new mutations either through evolution or selective pressure as a consequence of prolonged antibiotic exposure. This study suggests that the presence of TEM and CTX-M genes even the non-ESBL-producing \( E. \ coli \) can also pose a threat in the spread of antibiotic resistance because these strains serve as reservoir of ESBL-genes that will be developed through some mutations and needs to be included in strategies for detection and efforts at prevention of spread of ESBLs.

**CONCLUSION**

The presence of plasmid-mediated antibiotic resistance encoded by \( \beta \)-lactamase genes shows that these genes can be disseminated to bacteria of the same or different species, and can play a significant role in the mobility of resistance genes. Molecular detection which revealed a high percentage of CTX-M enzymes in ESBL-producing isolates also suggests that the spread of \( \text{bla}_{\mathrm{CTX-M}} \) gene is significantly contributing to the increase and prevalence of ESBL-producing \( E. \ coli \), thereby complicating treatment of bacterial infections. PCR-detection of the prevalent \( \beta \)-lactamase gene variants could be more valuable when coupled with nucleotide sequencing to detect specific ESBL derivative, not just for monitoring but more importantly for better therapeutic strategies. More isolates need to be evaluated to confirm the molecular characteristic of the \( \text{bla}_{\mathrm{SHV}} \) gene found in non-ESBL isolates. Moreover, the presence of ESBL-associated \( \beta \)-lactamase genes even in non-ESBL-producing \( E. \ coli \) suggests that these strains may serve as reservoirs for antibiotic resistance genes and may contribute to increased spread of antibiotic resistance among isolates, thereby posing a threat in the field of antibiotic therapy.

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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 AUTHORS**

Dr. Merlyn C. Cruz conceptualized the study, conducted the experiment, analyzed the data and prepared the manuscript. Dr. Cynthia T. Hedreyda contributed to data analysis and preparation of the manuscript.
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