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Molecular Detection of Extended Spectrum β-lactamase (ESBL) producing *Escherichia coli* from Chicken (*Gallus gallus domesticus*) meat isolates Public Markets in Cabanatuan City, Nueva Ecija

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Abstract. Antibiotic resistance poses considerable threat to human populations, especially those belonging to developing countries, like the Philippines. The high prevalence of improper antimicrobial usage has led to the rise of multi-drug resistant microorganisms, harboring genes, such as bla_{CTX-M}, responsible for the proliferation of microorganisms with extrinsic resistance to β-lactam antibiotics. This study investigated the presence of ESBL-producing *Escherichia coli* isolated from commercially available chicken meat sold in Public Markets from Cabanatuan City. Phenotypic confirmation of the species was done via macroscopic methods (culture) using selective and differential media supplemented with 1mg/L cefotaxime. Bacterial genomic DNA extraction using boil lysis method was done prior to PCR amplification using a primer for bla_{CTX-M} to confirm attributed to β-lactamase production. Nine (9) isolates were characterized, depicting dark colonies with green-metallic sheen in Eosin-Methylene Blue agar as *E. coli*. Presence of bla_{CTX-M} gene for ESBL-producing *E. coli* was detected across all isolates based on the ~592bp bands from the PCR amplification. Results of this study encompasses the need for surveillance of AMR, highlighting the significance of the risk posed by these resistant species present in food products. Further testing is recommended to fully characterize the isolates' resistance profiles and genotypes to further assess their geographical distribution.

Keywords: Antibiotic Resistance, Chicken meat, *E. coli*, Extended-spectrum β-lactamase

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1. Introduction

Antimicrobial resistance (AMR) continues to pose a significant global threat to the current state of our healthcare, making it one of the top public health concerns of the World Health Organization. With this concern, even the most common infection can become life-threatening and can possibly risk more lives. While AMR is typically caused by the overuse/misuse of antimicrobial agents, through the continued exposure and development of resistance, some bacteria acquire this resistance through horizontal gene transfers of conjugative plasmids (Crits-Christoph *et al.*, 2021). This means that the development of resistance poses more risk and potentially transfers this resistance to other microorganisms not known to cause any diseases.

Extended-spectrum β-lactamase are enzymes that have a known capability to degrade β-lactam rings in a variety of antibiotics, including (but not limited to) penicillins, cephalosporins, and cephamycins. This allows the microorganism to resist antimicrobials and are resistant to strong antibiotics, including extended-spectrum cephalosporins (Gundran, 2019). Since the discovery of ESBLs, several genes have been characterized to express β-lactamase production including the bla_{CTX-M} gene, which has been found dominant in most regions of the globe (Bevan *et al.*, 2017). This poses increased concerns over the presence of ESBL-coding genes, as even commensal species may harbor such virulence and easily transfer to other pathogenic bacteria.

It has been identified that broiler farms are prevalently known to supplement animals with various antimicrobial cocktails, contributing to the significant rise in antimicrobial resistance (Gundran *et al.*, 2019). Previous reports have already suggested these farms harbor multi-drug resistant pathogens due to improper use of antimicrobials, including the proliferation of ESBL-producing microorganisms. With this already previously established record of ESBLs in poultry farms seeming insignificant, the potential risk of transmission to humans increases, as meat and meat products now may be considered vectors. Several studies have already reported the contamination of ESBL-producing gram-negative bacteria in commercially available meat (Doi *et al.*, 2010; Overdevest *et al.*, 2010; Kang *et al.*, 2019), vegetables (Song *et al.*, 2020) and even in sampled wild game meat (Mateus-Vargas *et al.*, 2017; Sabenca *et al.*, 2021; Nüesch-Inderbinen *et al.*, 2022). Indications with these new studies now confirm that these farms are a possible source of ESBL-producing microorganisms, with

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genes easily being transferred from one organism to another, and easily transferred to humans through contamination of meat.

Despite the rigorous surveillance of antimicrobial resistance in the healthcare setting, there have been no previous record on the prevalence of ESBL-producing *E. coli* in meat products, confirming the hypothesis of transmission between broiler farms and consumers. Hence, this study aims to detect the bla_{CTX-M} gene expressing β-lactamase production in *E. coli* present in commercially available chicken meat, confirming the risk of transmission of pathogens harboring drug resistance from broiler farms to humans.

2. Methodology

2.1. Sampling Procedure

Bacterial isolation was carried out by using sterile cotton swabs pre-moistened with sterile saline, swabbed from the chicken meat and the pooled liquid underneath, and aseptically transferred to falcon tubes containing sterile saline (0.9% NaCl). Collection of samples shall be done in the early morning, from three (3) sampling sources: 1) Sangitan Public Market, Brgy. San Isidro; 2) Cabanatuan City Public Market, Brgy. Kapitan Pepe; and 3) a local 'Talipapa' in Brgy. Mabini Homesite (**Figure 1**). Sample tubes were then sealed to protect against contamination and placed on ice during transport. Microbiological analyses was done at the Microbiology Laboratory, College of Arts and Sciences, Nueva Ecija University of Science and Technology-General Tinio Campus.

2.2. Phenotypic Confirmation of Bacterial Isolates

Samples were streaked onto MacConkey agar plates supplemented with 1 mg/L Cefotaxime, to select for antibiotic-resistant gram-negative and differentiate members of lactose-fermenting Enterobacteriaceae, and were incubated aerobically at 37°C for 24 hours. Pink colony growth from the MacConkey agar, suggestive of lactose-fermenting bacteria and morphologically indicative of *E. coli* were picked and streaked onto an Eosin Methylene Blue Agar plate (EMB) and incubated at 37°C for 24 hours. EMB is also selective and differential. Distinctive metallic-green colonies are indicative of *E. coli*.

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2.3. Extraction of Genomic DNA

Bacterial genome extraction by boiling method was done by following the method described by Gundran (2019) with modifications. Bacterial colonies were suspended in 1000 µL sterile distilled water in a microcentrifuge tube and centrifuged at 4 500 rpm for 5 minutes. The supernatant was discarded and the bacterial cell pellets obtained were then washed with 100µL sterile distilled water and collected by centrifugation before resuspending the cells in 50µL sterile distilled water and putting them in a boiling water bath for 10 min. It was then cooled in crushed ice, and centrifuged at 1000 rpm for 5 minutes. The resulting bacterial lysate was stored at -20°C, which was used as template for PCR amplification.

2.4. Detection of blaCTX-M Gene Expressing β-lactamase Production

Polymerase Chain Reaction (PCR) amplifications was carried out using the optimized conditions of the specific primers used by Gundran (2019). The PCR assay was performed in a PCRmax Alpha Cycler-1 thermal cycler with the following amplification conditions: Initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 sec, and elongation at 72°C for 1 min with the final elongation at 72°C for 7 mins.

Table 1. The primer sequence of the target antibiotic resistance gene

Target Gene	Primer	Sequence (5'-3')	Amplicon size
bla_{CTX-M}	CTX-M-F	ATG TGC AGY ACC AGT AAR GTK ATG GC	592 bp
	CTX-M-R	TGG GTR AAR TAR GTS ACC AGA AYS AGC GG	

A total of 25 µL reaction volume containing one microliter (1µL) of DNA template and 0.5 µL of each primer was added to the reaction mixture containing OneTaq® Hot Start Quick-Load® 2X PCR Master Mix (New England BioLabs, USA). The resulting PCR products were then electrophoresed for 60 mins at 100V in 1.5% agarose gel (Cleaver Scientific, UK) stained with gel red nucleic acid dye (Biotium, USA) in 1X Tris-Borate-EDTA (Vivantis, Malaysia). To confirm the targeted molecular size, a 1kb DNA ladder (Bioline, Meridian Bioscience) was used as reference with an expected amplicon size of 592 bp.

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3. Results

3.1. Phenotypic Confirmation of Bacterial Isolates

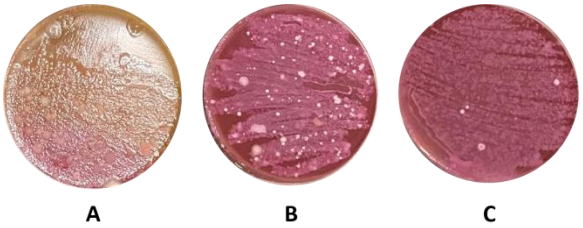

Figure 2. Isolates in MacConkey agar plates showing mixed cultures morphologies of both non-lactose fermenting and lactose fermenting enterobacteria. [A] Sangitan Public Market, [B] Cabanatuan City Public Market, [C] *Talipapa*

Nine *E. coli* isolated were confirmed by phenotypic assay in MacConkey agar with cefotaxime, showing lactose-fermentation and pink colonies. Phenotypic assay also revealed varying macroscopic culture characteristics, typical of several members of the Family Enterobacteriaceae (see **Figure 2**). All plates exhibited mix of lactose-fermenting (pink colonies) and non-lactose-fermenting species (yellow colonies). Confirmation of the presumed *E. coli* isolates was done by inoculating colonies in Eosin-Methylene Blue Agar, showing dark colonies with distinctive green-metallic sheen (see **Figure 3**).

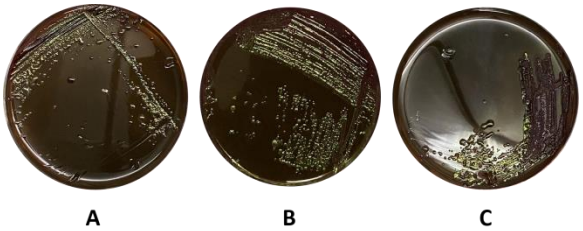

Figure 3. Isolates in Eosin-Methylene Blue agar plates showing distinctive green-metallic sheen from the colonies of *E. coli*. [A] Sangitan Public Market, [B] Cabanatuan City Public Market, [C] *Talipapa*

Further confirmation of the bacterial species, such as biochemical testing and sequencing, were not employed as culture cultivation methods proved enough to phenotypically determine the identity of the isolates. Despite this, it is recommended to employ such testing in future studies.

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3.2. Molecular Detection of ESBL-coding blaCTX-M gene in Escherichia coli

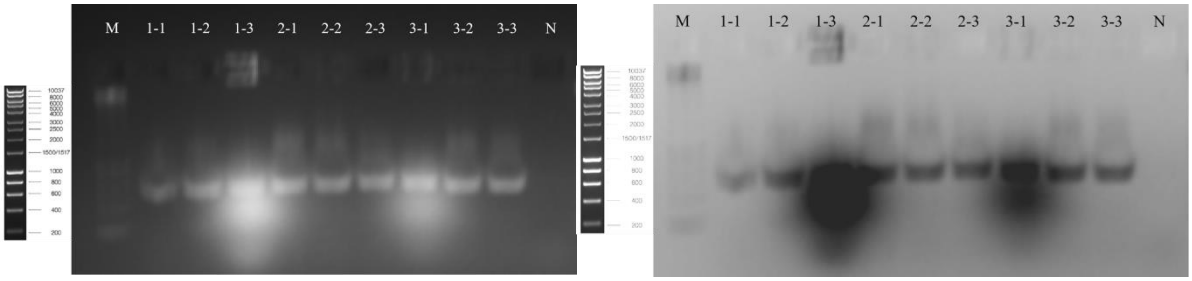

Figure 3. Electrophoresed PCR products, showing amplified bands at approximately ~592bp, compared to a 1kb DNA Ladder (M), and a Negative Control (N)

Genomic DNA extracted from the presumed *E. coli* bacterial isolates were tested for the presence of bla_{CTX-M} gene expressing β-lactamase production, showing the expected amplicon size of ~592bp across all isolates (as shown in Figure 3). This confirms the possible presence of ESBL-producing *E. coli* isolated from chicken meat.

4. Discussion

The use of both selective and differential media allows for the sensitive and specific detection of target microorganisms. Coupled with the addition of Cefotaxime, its selectivity for resistant species was increased. Similar results have been reported by previous authors expressing great concern over the significantly high prevalence of the presence of microorganisms with increased virulence (Díaz-Jiménez *et al.*, 2020; Gundran, 2019; Olsen, 2014; Blaak *et al.*, 2015). Antibiotic-supplemented media allows for the selection of presumed resistant strains but does not screen for resistance against other antibiotics or other genotypes. The use of molecular detection methods in this study provides high sensitivity and high specificity towards the detection of a gene responsible for the expression of β-lactamase production but is also generally reputed to be labor intensive, as well as expensive and not cost-efficient when compared to other routine methods of detection.

The detection of the bla_{CTX-M} gene does not necessarily indicate the presence of antibiotic-resistant microorganisms from chicken but suggests the resulting transmission of resistant bacteria from broiler farms and potentially poses higher risks during food preparation and handling. Previous studies detecting ESBL-producing Enterobacteriaceae in food and commercially available meat products have significantly reported the high prevalence of such organisms (Geser *et al.*, 2012; Evers *et al.*, 2017; Gundran *et al.*, 2019). Despite this, the

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detection of the bla_{CTX-M} gene from bacterial isolates causes great concern, as it serves as proof of the transmission of resistant *E. coli*, possibly harboring multi-drug resistance, to the commercial market and potentially transferring this resistance to other pathogenic microorganisms.

Due to the small sample size and sampling method, this detection provides less sensitivity to present an accurate representation of the entire poultry industry. While the results presented show a high isolation rate of resistant *E. coli*, considerations must be made when interpreting the results. One such way to more accurately represent the populations and draw more statistically conclusive results is to employ a higher sampling rate and more statistically reliable sampling method in future studies and contribute to the continuing surveillance of antimicrobial resistance in the country.

At the moment, it is still unclear whether the isolated species harbors other virulence factors contributing to its resistance. Further assessment of the organisms' resistance profile by expanding the representative types and classes of antibiotics used for antimicrobial resistance profiling is recommended. Furthermore, expanding the genotypic detection of other ESBL-expressing genes is recommended by multiplex PCR detection of other β-lactamase genes. While all the isolates in the present study exhibited the presence of the bla_{CTX-M} gene, other molecular mechanisms may also be at play contributing to the species' virulence. Hence, further genotyping is recommended to determine the specific sub-group of the isolated microorganism harboring the blaCTX-M gene and further elucidate its phylogenetic relationship with other strains to characterize its geographical distribution for continuous efforts of AMR surveillance and epidemiology.

5. Conclusions

The results of the study confirmed the presence of *Escherichia coli* from commercially available chicken meat, confirming the possible transmission of multi-drug resistant and other pathogenic species from broiler farms to commercial meat and meat products in marketplaces. Further characterization of its resistance profile and genotype is recommended to determine its source and possible geographical distribution.

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