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Using Quantitative Polymerase Chain Reaction (qPCR) to Identify a Myriad of Carbapenemase Genes in Fresh Cow Dung in Bangladesh

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Mamun Al Asad [1], Ayasha Siddique Shanta [1], Kakoli Akter [1], Marnusa Binte Habib [1], Shamsun Nahar [1], Mainul Haque [2, 3], Santosh Kumar [4], Salequl Islam [1, 5]

1. Microbiology, Jahangirnagar University, Dhaka, BGD 2. Dentistry, Karnavati University, Karnavati Scientific Research Center, Gandhinagar, IND 3. Pharmacology and Therapeutics, National Defence University of Malaysia, Kuala Lumpur, MYS 4. Periodontology and Implantology, Karnavati University, Karnavati School of Dentistry, Gandhinagar, IND 5. Optometry and Vision Science, University of New South Wales, Faculty of Medicine and Health, Sydney, AUS

Corresponding author: Salequl Islam, salequl@juniv.edu

Abstract

Introduction

The emergence of antimicrobial resistance (AMR) is driven by the selection pressure of frequent uses of antimicrobial agents in healthcare, the food chain, agriculture, fishery, and the food animal industry, which poses a serious health risk for transmission-linked humans and the surrounding environment. Livestock, particularly cattle, play an essential role in the food sector in Bangladesh. The food-animal chains can be the potential routes of exposure to AMR-microorganisms for every domain of one health. Antimicrobial resistance genes (ARGs) can impart a reservoir of AMR within the food supply chain, even without pathogenic microorganisms. This study investigated the history of infection for the last six-month period of antimicrobials utilized in cattle farms and the distribution of selected carbapenemase resistance genes, namely, *bla-KPC*, *bla-IMP*, *bla-VIM*, *bla-NDM-1*, *bla-SIM*, *bla-GIM*, *bla-SPM*, and *bla-SME*, in cattle feces in Bangladesh.

Methods

A cross-sectional study was designed to analyze ARGs in fresh cow dung samples collected from commercial farms and individual houses in four Bangladesh districts, namely, Dhaka, Gazipur, Manikganj, and Tangail. Types of cattle breeds, their existing diseases, recent antimicrobial uses, and vaccine uses were recorded. DNA was extracted from each cow dung sample using commercial kits (Qiagen GmbH, Germany). Real-time quantitative polymerase chain reaction (RT-qPCR) was employed to assess the eight carbapenem resistance genes in the extracted DNA. The eight carbapenem resistance genes in the extracted DNA were assessed by RT-qPCR using the qTOWER3 thermal cycler (Analytik Jena GmbH, Konrad-Zuse-Straße 1, 07745 Jena, Germany).

Results

Group A carbapenemase, *bla-KPC*, was detected in 66.7% of the samples. However, no *bla*-SME was identified in all of the test samples. Group B metallo carbapenemase, *bla-IMP*, *bla-NDM-1*, *bla-VIM*, *bla-SIM*, *bla-GIM*, and *bla-SPM*, were in 66.7% (80/120), 49.2% (59/120), 48.3% (58/120), 68.3% (82/120), 58.3% (70/120), and 12.5% (15/120), respectively. Only 8.3% of the tested samples contained no MBL gene; 10% carried a single-type carbapenemase gene; and the remaining 81.7% carried two or more carbapenemase genes concurrently. Co-carriage of four or more genes was found in over 59% of samples. As many as seven genes were found together in 6.7% of samples. ARG detection in commercial cattle samples and household feces is not statistically significant.

Conclusions

Substantial carbapenem-resistance ARGs were detected in commercially farmed cow dung and household cattle samples. Frequent use of antibiotics for cattle for treatment and prophylactic purposes may influence the high acquisition of ARGs. Bangladeshi cattle farms are reservoirs and routes of AMR, posing a significant threat to the country's public health.

Categories: Public Health, Infectious Disease, Environmental Health
Keywords: carbapenemase-resistant genes, bangladesh, fresh, polymerase chain reaction, measurable, cow dung, cattle farm, qpcr, mbl, args

Introduction

Antimicrobial resistance (AMR) is forecasted to be the leading cause of mortality, morbidity, and financial losses in the upcoming years [1]. Although the emergence of AMR is a natural property, it has been driven by

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the selection pressure of frequent uses of antimicrobial agents in healthcare, the food chain, agriculture, and fishery [2,3]. Considering the lower cost, availability, and safety, β-lactam antibiotics are currently the most commonly prescribed antimicrobials in humans [4] and cattle farming, particularly to treat bovine mastitis in dairy cow farms [5]. Both humans and animals circumvent the selective toxicity of the β-lactam antibiotics specific for bacterial peptidoglycans and transpeptidases [6]. β-lactam antibiotics belong to natural penicillin and hundreds of other penicillin derivatives such as monobactams, cephalosporins, cephamycins, and carbapenems [7]. Among the currently available β-lactams, carbapenems have become the reliable drug of choice because of their relatively high resistance to hydrolysis by most β-lactamases and robust target capacity for penicillin-binding proteins [8]. The clinically used carbapenems at present are imipenem, meropenem, ertapenem, doripenem, and panipenem, which are used parenterally to treat critical infections by both gram-positive and gram-negative aerobic and many anaerobic bacteria [9]. Reporting of carbapenem resistance has become a tremendous public health concern globally [10]. Several gram-negative bacteria (GNB), such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, are frequently reported to have carbapenem resistance [11], possibly by intrinsic mutations or mediated by transferable carbapenemase-encoding genes [10]. Almost 3,000 different β-lactam-hydrolyzing enzymes are reported [12], commonly called β-lactamases (designated as bla), and function as the primary driving force of resistance in GNB [11,13,14]. The well-known Ambler classification has grouped the β-lactamase enzymes into four molecular classes: A, B, C, and D, based on their primary sequence motif composition [15]. Group A, C, and D β-lactamases use serine at their active site amino acid motifs. At the same time, group B is called metallo-β-lactamases (MBLs) that bind one or two metallic zinc ions essentially at their active sites and aid in breaking down target antibiotics [15,16]. Group A β-lactamases can hydrolyze penicillins, classical cephalosporins, monobactam, imipenem, and meropenems and are further segregated into six subgroups, including *bla-KPC* (*Klebsiella pneumoniae carbapenemase*), *bla-GES* (Guiana extended-spectrum), and *bla-SME* (*Serratia marcescens* enzyme), based on other amino acid composition differences [17]. In contrast, the class B MBL group belongs to the *bla-IMP*, *bla-SPM*, *bla-VIM*, *bla-GIM*, *bla-NDM*, and *bla-SIM* and possesses a broad spectrum for the hydrolysis of almost all β-lactam antibiotics, with the exception of monobactams [18,19]. MBLs were first reported in the 1960s; however, MBL genes in GNB isolated from clinical infections and nosocomial outbreaks drew global attention in 1990 [20]. Since then, there has been increasing reporting of different MBL genes, namely, *bla-IMP*, *bla-VIM*, *bla-NDM*, and *bla-VIM* [21,22]. Reports of MBL gene identification and transmission were focused on clinical bacteria and hospital-associated infections for years [22]. Some recent studies have identified the dissemination of MBL genes from clinical to other environmental spheres [23,24]. Studying the carbapenem resistance gene distribution in the environment is worthwhile for its potential implications for human health and ecological nuisance. Products of cattle farms, such as cow dung and manure, could be classic reservoirs of metagenomic MBL genes or bacteria carrying those ARGs. To our knowledge, limited or no study has been undertaken to evaluate MBL gene prevalence in cow dung samples. This study investigated the prevalence of eight carbapenem resistance genes, *bla-KPC*, *bla-IMP*, *bla-VIM*, *bla-NDM-1*, *bla-SIM*, *bla-GIM*, *bla-SPM*, and *bla-SME*, in fresh cow dung from cattle farms and households in Bangladesh.

Materials And Methods

Study areas and sampling

A cross-sectional study was designed to collect fresh cow dung samples to assess the presence of MBL ARGs from December 2021 to December 2022. One hundred eight cow dung samples were collected from 20 commercial cattle farms. Twelve cow dung samples were collected from six individual houses. The history of current diseases and medicine usage was recorded using a brief, structured questionnaire. The sampling sites were chosen from some dairy farming areas located in four districts in Bangladesh, namely, Dhaka, Gazipur, Manikgang, and Tangail (Figure *1*). Following all safety precautions and aseptic techniques, cow dung samples were collected to avoid probable cross-contamination. Samples were taken in clean, pre-labeled stool specimen containers, stored immediately in insulated ice boxes, and transported to the One Health Laboratory of the Department of Microbiology, Jahangirnagar University, Savar, where subsequent molecular biology analyses were carried out.

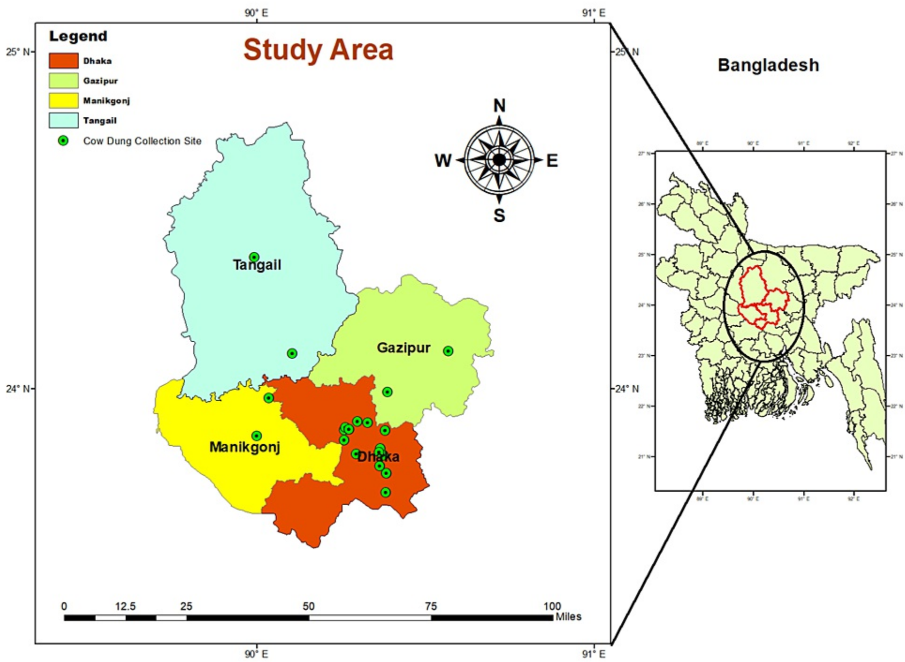

FIGURE 1: Sampling areas

Geographic locations of cattle farms (n=20) and houses (n=6) were shown from where cow dung samples were collected. Sampling sites include four districts in Bangladesh and are displayed on the map. Geographic information mapping software, ArcGIS version 10 for Windows (Esri, CA, USA), was used to draw the sampling site-location map.

DNA extraction from cow dung samples

Approximately one gram of each cow dung was suspended in 3 mL of sterile phosphate-buffered saline using a sterile spatula and mixed well. The QIAamp DNA stool mini kit (Qiagen GmbH, Germany) was used to manually extract DNA from resuspended cattle feces following the manufacturer's protocol. The quick purification of nucleic acids is made possible by the practical QIAamp spin-column method. The extracted DNA was eluted in 200 µl of elution buffer and preserved at -20°C for next-level analyses. Separate aliquots of extracted DNA were stored in a repository at -80°C for any future research.

Primer design

Eight pairs of MBL primer sequences (*bla-KPC*, *bla-IMP*, *bla-VIM*, *bla-NDM-1*, *bla-SIM*, *bla-GIM*, *bla-SPM*, *bla-SME*), their annealing temperatures, and amplicon lengths were obtained from previous literature [25]. The conformities of the oligonucleotide primer sequences were further checked in the NCBI BLAST (Basic Local Alignment Search Tool) database and then synthesized from an external manufacturer (Macrogen Inc., South Korea).

Real-time polymerase chain reaction amplification program

Real-time quantitative polymerase chain reaction (RT-qPCR) was used to determine relative (per 16S rRNA gene) abundances of ARGs in DNA extracted from cow dung samples. The qTOWER3 thermal cycler (Analytik Jena, GmbH, Germany) was used to amplify the qPCR. For each qPCR reaction, extracted DNA 1.0 µl was added to 10 µl of Go Taq qPCR master mix (Promega Corporation, 2800 Woods Hollow Rd, Fitchburg, WI 53711, United States), and five µmol of each primer (2 µL), and the nuclease-free water was added to make a final volume of 20 µL. The optimal program of the qPCR includes an initial denaturation at 95°C for two minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds; an annealing temperature of 58°C was used to amplify genes *bla-KPC*, *bla-IMP, bla-VIM,* and *bla-NDM-1* and 56°C for the genes *bla-SIM, bla-GIM, bla-SPM*, and *bla-SME* for 15 seconds and an extension at 60°C for 20 seconds. The amplification process was completed with a melting step that was carried out using the following cycling parameters: 60°C for 15 seconds and 5°C temperature adjustments up to a final temperature of 95°C. SYBR green's fluorescence energy was used to measure the amount of amplified product. SYBR green binds double-stranded DNA after extension by PCR and emits fluorescence. A negative control (no template control) was used in each PCR run. The bacterial 16S rRNA gene was used as a positive control.

Determination of specificity and sensitivity of the qPCR

The following equations were used to calculate specificity and sensitivity: specificity = [D/(C + D)]*100 and sensitivity = [A/(A + B)]*100, where A is a true positive, B is a false negative, C is a false positive, and D is a true negative (Table 1). The standard curve approach obtained the correlation coefficients' R2 values. All the genes had R2 values of 0.98.

ARG analyzed	True (+) = A	False (-) = B	False (+) = C	True (-) = D	% sensitivity; specificity*
bla-KPC	80	0	0	40	100; 100
bla-IMP	80	0	0	40	100; 100
bla-NDM-1	59	0	0	61	100; 100
bla-VIM	58	0	0	62	100; 100
bla-SME	0	0	0	120	100; 100
bla-SPM	15	0	0	105	100; 100
bla-GIM	70	0	0	50	100; 100
bla-SIM	82	0	0	38	100; 100

TABLE 1: Specificity and sensitivity of the qPCR detection system for carbapenemase genes

%, percentage; A, true positive; B, false negative; C, false positive, D, true negative. The sensitivity and specificity of each carbapenem gene detection were determined based on the negative control results. No template was added for real-time quantitative polymerase chain reaction (RT-qPCR) amplification and negative control results, where known bacterial 16S rRNA gene was used with respective primer pairs

Statistical analysis

Descriptive and inferential statistics determined the MBL genes in different cow dung samples. Descriptive statistics were reported as frequencies and percentages. Pearson's chi-square test was used to test the association between ARG carriage in farm-based cow dung and household cow dung. A two-tailed p-value of 0.05 or smaller was considered statistically significant. SPSS Statistics version 20.0 (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) was used to analyze all the data.

Ethics statement

This study was an extension of a similar previous research project approved by the Ethics and Research Review Committee of the Jahangirnagar University Faculty of Biological Sciences (approval number: BBEC, JU/M 2017 12(4), approval date: December 27, 2017). The ethical committee has waived the requirement for new approval. The study followed all the ethical guidelines and regulations for environmental samples. Informed verbal consent was obtained from each cattle owner/farm manager for collecting the cow dung samples and information from their farms/cattle. Farm identities were kept anonymous to protect commercial, personal, and private information. A sample identification code was assigned correctly for each sample collected.

Results

Study farms and samples

Out of 120 samples, 108 (90%) cow dung samples were collected from 20 commercial cattle farms. The farms were rearing a mixture of dairy and beef-producing cattle, namely, Holstein Friesian, Holstein Friesian Jersey, Sahiwal, and their breeds. Some farms raise well-known indigenous breeds like Red Chittagong cattle, Munshiganj cattle, and Pabna cattle. Twelve cow dung samples (10%, 12/120) were collected from individual houses raising non-described local cattle. Two cattle were foot-rot-infected, and seven had fevers during the sample collection. All except four cattle had antibiotic exposure within the last six months. The cattle were being treated with homeopathy for years. Antibiotic use history was almost similar between the cattle of commercial farms and backyard farms. Over 96% (116/120) of the cattle were vaccinated for either anthrax, foot and mouth diseases, or black quarter.

Distribution of carbapenemase genes in cow dung samples

This study measured the presence of eight critical carbapenemase genes in 120 cow dung samples collected from 20 Bangladeshi cattle farms and six individual houses. We found only 10 cow samples where no MBL gene was detected; the remaining 110 samples were detected with at least one MBL gene out of eight genes

investigated. Group A carbapenemase, *bla-KPC*, was detected in 66.7% of the samples. However, *bla-SME* was identified in all of the test samples. In contrast, Group B metallo carbapenemases, *bla-IMP*, *bla-NDM-1*, *bla-VIM*, *bla-SIM*, *bla-GIM*, and *bla-SPM*, were in 66.7% (80/120), 49.2% (59/120), 48.3% (58/120), 68.3% (82/120), 58.3% (70/120), and 12.5% (15/120), respectively (Table 1). The newly optimized qPCR technique identified all the carbapenemase genes in the test samples. We calculated the sensitivity and specificity of each carbapenem gene detection by RT-qPCR cycle. Deionized water was used as the negative control template, and a known bacterial 16S rRNA gene was used with respective primer pairs as the positive control. A cut-off cycle threshold (Ct) value of 30 was determined to be selected as ARG-detection positive. No expected deviation was found in amplification cycles for positive and negative controls. Hence, the qPCR's specificity and sensitivity were 100% (Table 1).

Only 10% of the tested samples carried single-type carbapenemase genes; the remaining 81.7% carried two or more carbapenemase genes concurrently. Co-carriage of four or more genes was found in over 59% of samples. As many as seven genes were found in 6.7% of samples (Figure 2).

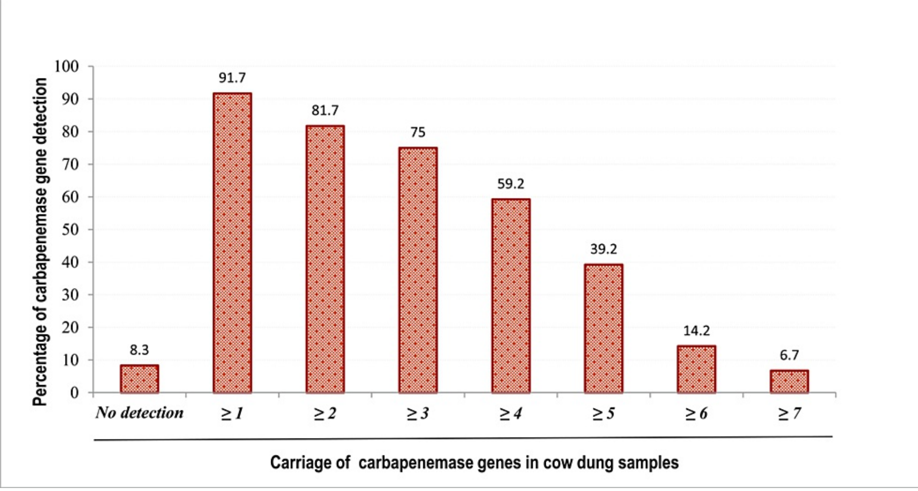

FIGURE 2: Cumulative distribution of carbapenemase genes in cow dung samples

The value bar shows the percentage prevalence. Only 8.3% where no metallo-β-lactamase (MBL) gene was detected, 10% of the tested samples were found to carry single type carbapenemase gene, and the remaining 81.7% of samples were carrying two or more carbapenemase genes concurrently. Co-carriage of four or more genes was found in over 59% of samples. As many as seven genes were found together in 6.7% of samples

Comparative distribution of carbapenemase genes in farm-based and household cow dungs

From the assessment of 108 cow dung samples from commercial farms, the prevalence of *bla-KPC*, *bla-IMP*, *bla-NDM*, *bla-VIM*, *bla-SPM*, *bla-GIM*, and *bla-SIM* was detected in 69.4%, 67.6%, 50.9%, 46.3%, 13%, 55.6%, and 70.4%, respectively. No significantly different prevalences of the genes were noticed in household cow dung samples. Five genes, *bla-KPC*, *bla-IMP*, *bla-NDM*, *bla-SPM*, and *bla-SIM*, were higher in commercial samples, and the other two genes exhibited the reverse prevalence (Figure 3).

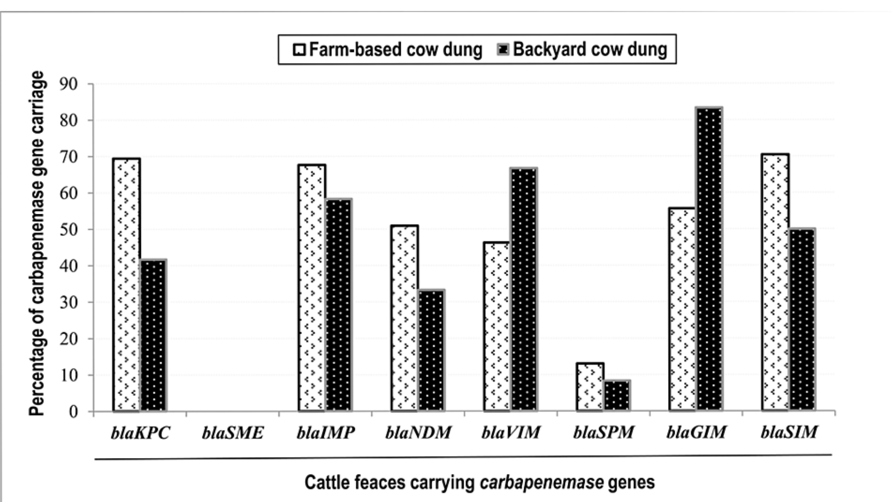

FIGURE 3: Comparative distribution of carbapenemase genes in farm-based and household cow dung samples

Five carbapenem resistance genes, *bla-KPC, bla-IMP, bla-NDM, bla-SPM*, and *bla-SIM*, were found to be higher in commercial samples. The two carbapenem resistance genes, *bla-VIM* and *bla-GIM*, were exhibited higher in household cow dung samples. The differences in antimicrobial resistance gene (ARG) prevalences are not statistically significant. One Group A carbapenem resistance gene, *bla-SME*, was neither identified in commercial cow dung samples nor household cow dung samples

Discussion

We investigated the contamination of AMR traits, focusing on eight carbapenemase genes, in both commercial cattle farm-based cow dung and home-based backyard farming cattle cow dung collected from four districts of Bangladesh. The study showed that over 90% of the tested cow dung samples carried some hazardous carbapenemase gene. Carriage of both serine-based Grou A and zinc metal-based Group B carbapenemase (MBL) genes was there. The prevalence of the ARGs in the cow dung samples varied substantially, with no detection for *bla-SME* and low detection for *bla-SPM*. The *bla-SME* and *bla-SPM* were reported as less common minor carbapenemase genes along, with *bla-GIM* and *bla-SIM* [26,27]. Unexpectedly, our study has identified a high prevalence of *bla-GIM* and *bla-SIM* together with the classical carbapenemase genes *bla-IMP*, *bla-NDM*, and *bla-VIM* [22,28].

More than half of the samples had four or more carbapenemase genes together. Some earlier studies reported some ARGs of β-lactam resistance, quinolone resistance, and colistin resistance in organic manures [29]; however, reporting the carbapenem resistance gene at this high abundance has not been reported yet. The abundance of carbapenem-resistance genes in Bangladeshi animal husbandry substantiates earlier studies stating that Indian subcontinental countries are endemic with carbapenemase genes in the human-animal-environment interface [22,30]. The MBL-encoding genes are generally transmitted by mobile genetic elements, facilitating rapid horizontal gene transfer (HGT) among different bacteria [28]. Therefore, our identified MBL genes may be disseminated from the cow dung reservoir to other domains of One Health, which link the health of animals, humans, plants, and environments in an interdependent cycle [31]. The high concentration of AMR hazards can easily be exposed to humans via direct or indirect contact with the climate, soil, agriculture, or food and water cycles. When these ARGs containing cow dung are disposed of in water or a nearby environment or used for agriculture/garden manure, there is a high possibility of spreading the ARGs to other pathogens through vertical or HGT. This study has set a surveillance framework for ARG screening tests in microbiology culture-free settings by rapid RT-qPCR techniques. The study framework is considered a step toward the sustainable development goal of surveillance of AMR under the One Health approach for developing countries, as emphasized by the tripartite alliance of the World Health Organization, the Food and Agriculture Organization, and the World Organization for Animal Health [32].

When adopting this study, we hypothesized that commercial farm-origin cow dung would be a reservoir of higher carbapenem resistance genes than household-origin cow dung. Our study did not find significant differences in carbapenem resistance genes in terms of abundance and diversity in the two samples. Our study has identified that the uses of antibiotics for cattle in commercial farms and individual houses are almost similar. Cephalosporin-group antibiotics were found to be the drug of choice for both cattle farms and household settings. As all the antibiotics are available over the counter in Bangladesh, people frequently buy and consume antibiotics in their jurisdiction. Used antibiotic sachets and parts of unused antibiotics are commonly disposed of in nearby household garbage. These discrepantly exposed antibiotics may contribute to the high acquisition of ARGs in food-producing animals and the surrounding environments [33,34]. As

reported, reducing antimicrobial usage often results in diminished AMR emergence [35], and some policies are imperative to reduce the AMR burden in cow dung.

Compared to traditional phenotypic and conventional approaches, a qPCR system provides a quicker and more effective technology for determining antibiotic susceptibility. We looked into the possibility of identifying significant genes for antibiotic resistance in cow dung samples without culture. Our qPCR system has the advantage of detecting the target ARGs with 100% sensitivity and 100% specificity, as affirmed by earlier studies [36,37].

Limitations of the study

This study had a few common limitations. The study was conducted under a cross-sectional study design, and a follow-up assessment was not carried out due to resource limitations. This study did not assess the acquisition of ARGs in bacterial communities by microbiological investigation. Future research will be crucial to determining the harborage of ARGs in bacteria and their associated phenotypic antimicrobial susceptibilities. This study analyzed the distribution of eight carbapenem-resistance genes, leaving behind a few unrelated gene variants that were not investigated. The small sample size remains an inherent constraint to conducting well-strength statistical analyses. However, our studies generated data by maintaining internal validity by repeating independent experiments when necessary. We believe the findings shed some light on the potential source of disseminating critical carbapenem resistance genes at the animal-human interface.

Conclusions

To the best of our knowledge, our study successfully implemented the first qPCR assays to identify carbapenem resistance genes in Bangladeshi food at animal-human interfaces. Thus, our current efforts have laid the foundation for surveillance of ARG detection at the animal-human interface; the approach should be continued with comprehensive coverage. Our study provides the groundwork for doable, simple, and affordable qPCR-based surveillance of AMR that could be conveniently implemented in low- and middle-income countries. The study design can be further expanded for other One Health domains. To preserve the efficacy of carbapenems as last-resort antibiotics, further detailed AMR surveillance and associated national policy are urgently required to focus on carbapenem resistance.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

Concept and design: Mainul Haque, Mamun Al Asad, Ayasha Siddique Shanta, Marnusa Binte Habib, Shamsun Nahar, Santosh Kumar, Salequl Islam, Kakoli Akter

Acquisition, analysis, or interpretation of data: Mainul Haque, Mamun Al Asad, Ayasha Siddique Shanta, Marnusa Binte Habib, Shamsun Nahar, Santosh Kumar, Salequl Islam, Kakoli Akter

Drafting of the manuscript: Mainul Haque, Mamun Al Asad, Ayasha Siddique Shanta, Marnusa Binte Habib, Shamsun Nahar, Santosh Kumar, Salequl Islam, Kakoli Akter

Critical review of the manuscript for important intellectual content: Mainul Haque, Mamun Al Asad, Ayasha Siddique Shanta, Marnusa Binte Habib, Shamsun Nahar, Santosh Kumar, Salequl Islam, Kakoli Akter

Supervision: Mainul Haque, Mamun Al Asad, Ayasha Siddique Shanta, Marnusa Binte Habib, Shamsun Nahar, Santosh Kumar, Salequl Islam, Kakoli Akter

Disclosures

Human subjects: All authors have confirmed that this study did not involve human participants or tissue. **Animal subjects:** This study was an extension of a similar previous research project approved by the Ethics and Research Review Committee of the Jahangirnagar University Faculty of Biological Sciences (No. BBEC, JU/M 2017 12(4), dated December 27, 2017). The ethical committee has waived the requirement for new approval. The study was conducted following all the ethical guidelines and regulations for environmental samples. Informed verbal consent was obtained from each cattle owner/farm manager for collecting the cow dung samples and information from their farms/cattle. Farm identities were kept solely anonymous to protect their commercial, personal, and private information. A sample identification code was assigned duly for each sample collected. Issued protocol number No. BBEC, JU/M 2017 12(4). **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial

relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

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Culture-Independent Quantitative PCR Detected Mobilized Colistin Resistance Genes (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5*) in Chicken Gut Contents in Bangladesh

Mamun Al Asad [1], Md Sarower Hossen Shuvo [1], Shomaia Yasmin Mitu [1], Sumia [1], Md Asief Hossain Zihadi [2], Ayasha Siddique Shanta [1], Nahidul Islam [1], Shamsun Nahar [1], Brian Godman [3,4] and Salequl Islam [1,5,*]

[1] One Health Laboratory, Department of Microbiology, Jahangirnagar University, Savar, Dhaka 1342, Bangladesh; ronniebge22@gmail.com (M.A.A.); sarowerhossen9@gmail.com (M.S.H.S.); shomaiamitu@gmail.com (S.Y.M.); sumiaalam26@gmail.com (S.); shantamicro44@gmail.com (A.S.S.); nahidulmicro44@gmail.com (N.I.); nahar@juniv.edu (S.N.)
[2] Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka 1342, Bangladesh; asief@blri.gov.bd
[3] Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow G4 0RE, UK; brian.godman@strath.ac.uk
[4] Division of Public Health Pharmacy and Management, School of Pharmacy, Sefako Makgatho Health Sciences University, Pretoria 0204, South Africa
[5] School of Optometry and Vision Science, Faculty of Medicine and Health, UNSW Sydney, Sydney, NSW 2052, Australia
* Correspondence: salequl@juniv.edu; Tel.: +880-1715029136; Fax: +880-2-7791052

Abstract: Inappropriate antimicrobial use in food animal farming propels antimicrobial resistance (AMR) that affects all health domains. Colistin is a 'Reserve' antibiotic for human treatment to be conserved for multidrug-resistant pathogens; however, it is being used as an animal growth promoter in many developing countries. The evolution of mobilized colistin resistance (*mcr*) gene-mediated colistin resistance has been reported to be associated with rampant colistin use. This study investigated the current variants of the *mcr* gene in chicken gut contents in Bangladesh. A cross-sectional study was designed to assess the *mcr-1* to *mcr-5* genes in 80 fresh poultry droppings from commercial poultry farms and 40 poultry droppings from household farms. DNA was extracted from each poultry dropping using commercial kits (Qiagen GmbH, Hilden, Germany). Real-time quantitative polymerase chain reaction (RT-qPCR) was employed using the qTOWER3 thermal cycler (Analytik Jena GmbH, Jena, Germany) to analyze the *mcr* gene variants in the extracted DNA. This study observed that 47.5% (57/120) of the samples exhibited the presence of at least one *mcr* gene out of the five variants investigated. The individual detection rates of the *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* genes were 42.5% (51/120), 2.5% (3/120), 1.7% (2/120), 5% (6/120), and 9.2% (11/120), respectively. The co-carriage of two or more genes was found in over 10% (10/57) of the samples. The triple occurrence of *mcr* genes was identified in three samples with the combination of *mcr-1*+*mcr-2*+*mcr-4*, *mcr-1*+*mcr-3*+*mcr-5*, and *mcr-1*+*mcr-4*+*mcr-5*. Overall, a significantly higher number of *mcr* genes were identified in the commercial farm chicken droppings compared to the household chicken droppings (p = 0.007). The existence of *mcr* genes in poultry feces in Bangladesh emphasizes the importance of proper poultry waste disposal and good hygiene practices in poultry livestock and its value chain. The potential impact of environmental ARGs should be considered in national and global policy documents. An integrated and combined approach to the One Health concept should be applied in all domains to understand and control the environment's role in the evolution and transmission of AMR.

Keywords: antimicrobial resistance genes (ARGs); mobilized colistin resistance genes (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5*); qPCR; Bangladesh

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1. Introduction

The demand for animal protein for humans has been increasing worldwide over the past few decades [1]. By 2033, the global requirements of poultry, sheep meat, pig meat, and beef are calculated to increase by 16%, 16%, 11%, and 8%, respectively, reaching a projected retail consumption weight equivalent to 28.6 kg/year/person [2]. The increased demand for animal protein production currently requires the increased application of antimicrobials in food animal farming [3]. The World Organization for Animal Health (WOAH) has estimated that an average of 99.1 to 108.5 milligrams of antimicrobials were used per kilogram of animal biomass covering bovine, swine, poultry, and aquatic species in 2019 [4], with a marginal increase in 2021 up to 109.65 milligrams [5]. Over 70% of all antimicrobials are used for animals worldwide [6,7]. Inappropriate antimicrobial use in food animal farming propels antimicrobial resistance (AMR), affecting all the domains of health including animal health, the environment, and human health [6,8,9]. Overall, the use of antibiotics outside the healthcare sector is considered one of the single most important factors leading to AMR [10–13]. The estimated global antimicrobial usage was 99,502 tons in 2020 and is projected to increase by 8.0–11.5% by 2030 unless it is addressed [3,7]. Asia/Oceania remain the key continents with considerable concerns regarding their antimicrobial use in animals, although all continents are prone to increased use [7]. Tetracyclines are currently the most utilized antimicrobial agent in animal health worldwide (35.5% of the total usage) followed by penicillins (13.3%) [4]. Both of these antimicrobials belong to the Veterinary Critically Important Antimicrobial (VCIA) classes and are not considered critically important antimicrobials for human health as they both belong to the 'Access' group of antibiotics [14,15]. This is unlike colistin, which is classified as a 'Reserve' antibiotic for human treatment to be conserved for multidrug-resistant pathogens [15,16]. Colistin, once widely used in livestock feed for growth promotion, has faced increasing regulatory scrutiny due to concerns over antibiotic resistance [17]. In the European Union, its use has been banned since 2016, allowing only for therapeutic application to protect public health [18]. Similarly, in the United States, the FDA prohibits colistin in animal feed, emphasizing the need to preserve its effectiveness in human medicine [19]. Other countries are also tightening their regulations, reflecting a global trend toward restricting the use of critically important antibiotics in agriculture [20]. Until 2019, colistin was permissible in livestock and poultry industry as a feed additive and therapeutic. In 2019, the Directorate General of Drug Administration (DGDA) Bangladesh banned all combined colistin preparations from veterinary use by canceling the registration of such products [21]. However, because of weak legislative monitoring, some levels of colistin were found to be used in animal husbandry and agriculture in several countries [4,22]. Colistin is a polymyxin family antibiotic, a natural cationic polypeptide that binds the lipid A of lipopolysaccharides (LPSs) in the outer membrane of Gram-negative bacteria [23,24]. This antimicrobial agent was discovered in 1947 and was considered a low-risk antibiotic for AMR gene acquisition and transmission by the European Medicines Agency (EMA) until 2015 [25]. The acquisition and transferability of the plasmid-mediated mobilized colistin resistance gene variant-1 (*mcr-1*) was first reported in Enterobacterales bacteria from animal products and humans in China [26] in 2016 and subsequently became omnipresent in many countries [27,28]. To date, ten slightly different genotype variants of the transferable *mcr-1* gene (*mcr-1* to *mcr-10*) have been reported in different bacterial isolates from pigs, bovines, poultry, food, humans, and the environment [25,29,30]. China currently uses the greatest volume globally of antibiotics in the livestock and poultry industries [4], with polymyxins extensively used as growth promoters for disease prophylaxis in China until 2016 [31]. As a result, a surge in *mcr* gene-mediated colistin resistance has been reported, which authenticates the relationship between antibiotic use and AMR evolution [32,33]. Since 2017, colistin has been banned in China as an animal growth promoter [34]. Encouragingly, the use of colistin as a growth promoter has now been curtailed in over 50% of developed countries in the last five years up to 2021 [4], with such measures found to be effective in reducing the evolution of colistin-resistant bacteria [35–37]. However, polymyxins are still being used in developing countries despite

these being listed as "highest priority critically important antimicrobials" (Highest Priority CIA by the WHO) for human medicine [4,14,38]. Of growing concern is the continually high prevalence of *mcr* gene variants observed in livestock farming, mainly among poultry and pigs [25,31,39]. *mcr* genes are worrisome globally because of their rapid interspecies spreading capacity via horizontal gene transfer mechanisms [40,41]. The transferability of the *mcr* genes from animal bacteria to human pathogens has also been seen during in vitro analyses [26,42], with colistin-resistant pathogens seen to account for higher mortality among critically ill patients with concerns regarding the lack of available options for these patients [43–46]. Consequently, the identification of the primary source, associated factors, and transmission of colistin resistance are key global priority areas. However, traditional detection methods such as the conventional polymerase chain reaction (PCR) and Sanger sequencing are time-consuming and labor-intensive for *mcr* gene analyses. Various real-time quantitative PCR methods have recently been devised for the more rapid and specific identification of *mcr* gene variants [47,48]. This is of considerable importance in Bangladesh to help guide future policies regarding the appreciable availability and use of antibiotics without prescriptions, including 'Reserve' antibiotics such as polymyxins [14,49–51]. Consequently, there is a need to investigate the current different variants of the *mcr* gene in chicken gut contents in Bangladesh using culture-independent RT-qPCR techniques. This also includes the development of a rapid and sensitive real-time PCR method for the specific detection of all five *mcr* genes in fresh gut samples from poultry farms and native sources in Bangladesh. These were the objectives of this study. The findings of this research are important for understanding the most important reservoir of *mcr* genes and their abundance in Bangladesh and for providing future guidance. Culture-independent ARG detection in chicken gut contents will build the foundation for future studies to predict comprehensive chicken gut resistant microbiota that are potentially disseminated in all the One Health domains. The findings can be used to develop future policies in Bangladesh and beyond, where there are concerns.

2. Materials and Methods

2.1. Study Area and Sampling

A cross-sectional study was designed to assess the presence of *mcr* genes in fresh chicken guts between January 2021 and November 2021. Eighty fresh poultry droppings were collected from 16 commercial poultry farms and forty poultry droppings were collected from 20 separate household farms. Information regarding the history of diseases and medication use was obtained through a structured questionnaire developed and validated in our previous study [52]. Sampling sites were selected from poultry farming areas across five districts in Bangladesh: Dhaka, Gazipur, Manikgang, Tangail, and Mymensingh (Supplementary Figure S1). All necessary safety measures and aseptic techniques were followed during sample collection to prevent potential cross-contamination. The samples were placed in clean, labeled containers and promptly stored in insulated ice boxes. These were then transported to the One Health Laboratory at the Department of Microbiology, Jahangirnagar University, Savar, where subsequent molecular biology analyses were conducted.

2.2. DNA Extraction from Chicken Gut Samples

Approximately one gram of each chicken dropping sample was mixed thoroughly with 3 mL of sterile phosphate-buffered saline (PBS) using a sterile spatula. DNA extraction from the resuspended chicken stools was performed manually using the QIAamp DNA stool mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. This kit utilizes the QIAamp spin-column method for the quick purification of nucleic acids. Following extraction, the DNA was eluted in 200 µL of elution buffer and stored at −20 °C for subsequent analyses. Additionally, separate aliquots of the extracted DNA were preserved in a repository at −80 °C for potential future research endeavors.

2.3. Primer Design for Assessing ARGs

The primer sequences for five pairs of *mcr* genes (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5*) along with their corresponding annealing temperatures and amplicon lengths were acquired from previous literature [47,48]. These primer sequences were then cross-referenced for accuracy using the NCBI BLAST (Basic Local Alignment Search Tool) database (Table 1). Subsequently, the primers were synthesized by an external manufacturer (Macrogen Inc., Teheran-ro, Seoul, South Korea).

Table 1. Primers and positive controls used for the detection of *mcr-1* to *mcr-5* and *16S* genes in qPCR assays.

Primers	Sequences (5′-3′)	Amplicon Size (bp)	References
mcr-1-qf [a]	AAAGACGCGGTACAAGCAAC	213	[47,48]
mcr-1-qr [b]	GCTGAACATACACGGCACAG		
mcr-2-qf	CGACCAAGCCGAGTCTAAGG	92	[47,48]
mcr-2-qr	CAACTGCGACCAACACACTT		
mcr-3-qf	ACCTCCAGCGTGAGATTGTTCCA	169	[47,48]
mcr-3-qr	GCGGTTTCACCAACGACCAGAA		
mcr-4-qf	AGAATGCCAGTCGTAACCCG	230	[48]
mcr-4-qr	GCGAGGATCATAGTCTGCCC		
mcr-5-qf	CTGTGGCCAGTCATGGATGT	98	[48]
mcr-5-qr	CGAATGCCCGAGATGACGTA		
16S-qf [c]	CGGTGAATACGTTCYCGC	467	[48,53]
16S-qr	GGWTACCTTGTTACGACTT		

[a] indicates the forward primer of the mobilized colistin resistance gene. [b] demonstrates the mobilized colistin resistance gene's reverse primer. [c] shows the specified primer for the 16s RNA used as an internal control in qPCR.

2.4. Optimization of qPCR Conditions for mcr-1 to mcr-5 Assays

Real-time quantitative polymerase chain reaction (qPCR) was utilized to assess the relative abundances of antimicrobial resistance genes (ARGs) per 16Sr RNA gene in DNA extracted from chicken dropping samples. The qTOWER3 thermal cycler (Analytik Jena GmbH, Jena, Germany) was employed for qPCR amplification. In each qPCR reaction, 1.0 μL of extracted DNA was combined with 10 μL of Go Taq qPCR master mix (Promega Corporation Inc., Fitchburg, WI, USA), 2 μL of each primer, and nuclease-free water to reach a final volume of 20 μL. The optimal qPCR program involved an initial denaturation at 95 °C for 2 min, followed by 45 cycles of denaturation at 95 °C for 15 s. An annealing temperature of 53 °C was used to amplify genes *mcr-1*, *mcr-2*, and *mcr-3*, and 55 °C for genes *mcr-4* and *mcr-5*, each for 15 s, followed by extension at 60 °C for 20 s. The amplification was finalized with a melting step, cycling through temperatures from 60 °C for 15 s, with adjustments up to a final temperature of 95 °C. SYBR Green fluorescence was utilized to quantify the amplified product.

2.5. Efficiency and Validation of qPCR Assays

A standard curve method was used to measure the effectiveness of the amplicon, according to Rutledge and Cote [54]. To assess the efficacy and dynamic range of each primer pair, serial dilutions ranging from 10^{-1} to 10^{-5} were prepared using DNA templates of varying concentrations (median concentration: 14.2 ng/μL, minimum: 0.4 ng/μL, maximum: 29.6 ng/μL). The concentrations of DNA were measured using a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Using melting profile analysis, the amplification plots' validity was evaluated. Additionally, to confirm the validity of the assays, both a positive (*mcr*+) and a negative (*mcr*-) control were included for each gene in every qPCR cycle. The 16S rRNA gene served as an internal process control and facilitated the standardization of the counts of *mcr-1* to *mcr-5* gene copies during the analysis of ARGs in chicken dropping samples [48].

2.6. Statistical Analysis

Descriptive and inferential statistical methods were applied to assess the presence of *mcr* genes across various chicken gut samples. Descriptive statistics were utilized to present the frequencies and percentages of occurrence. Pearson's chi-square test was employed to examine the potential association between the carriage of antimicrobial resistance genes (ARGs) in commercial poultry droppings and that from household settings. A significance threshold of 0.05 or lower for the two-tailed p-value was adopted to determine statistical significance. The analysis of all data was conducted using IBM SPSS version 20.0.

3. Results

3.1. Study Farms and Samples

From a total of 120 poultry droppings, 80 (66.7%) were obtained from chicken droppings across 16 commercial poultry farms. These farms housed a variety of chicken breeds for both egg and meat production, including Broilers, Layers, and Sonali variants. Additionally, 40 samples (33.3%) were collected from the chickens in 20 individual houses. Among the farm chickens, 16 were diagnosed with fowl cholera, 13 with infectious coryza, and several others showed signs of illness during the sampling period. All the flocks had been exposed to antibiotics within the past 3 months. In the case of the backyard chickens, 38 droppings (95%, 38/40) showed no history of antibiotic exposure. Another 5% (2/40) of the chickens had received antibiotic treatment within one month prior to sampling. The commercial poultry farms were found to use various types of antibiotics, notably, amoxicillin, oxytetracycline, gentamicin, ciprofloxacin, and colistin, in different combinations in higher proportions from 12.5% to 37.5%. Only 5.0% of the household chickens were reported to receive oxytetracycline, but no other antibiotics. Colistin was reported to be administered to 12.5% of the commercial poultry chickens (Table 2). Overall, a statistically significantly higher antibiotic usage was recorded in the commercial farm chickens compared to the household chickens (p = 0.000).

Table 2. Antibiotic usage history in chickens during the past three months.

History of Antibiotic Usage in the Chickens over the Last Three Months	Types of Chickens	
	Poultry (n = 80), Number (%)	Household (n = 40), Number (%)
Oxytetracycline	30 (37.5)	2 (5.0)
Amoxicillin and oxytetracycline	14 (17.5)	0
Amoxicillin, gentamicin, oxytetracycline, and ciprofloxacin	26 (32.5)	0
Colistin, oxytetracycline, ciprofloxacin, and amoxicillin	10 (12.5)	0
No major antibiotic used	0	38 (95.0)

%, percentage.

3.2. Analytical Performance of mcr-1 to mcr-5 RT-PCR Assays

We used the optimized amplification settings for each of the *mcr* genes. The assays' efficiencies ranged from 97.3% to 105.3%, and each R^2 value was >0.98 (Figure 1). The maximum limit number of quantification copies was 1.89×10^3, 8.03×10^2, 1.60×10^3, 2.37×10^2, and 4.36×10^2 for the *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* assays, respectively. Using a melting plot analysis, the qPCR yielded a clean melting curve with only one peak and without interference from contaminants or non-specific amplification (Figure 1). No instances of primer dimers or non-specific signals were observed in any of the assays. Whether utilizing template references or employing *mcr* oligonucleotide pairs as opposed to non-target *mcr* genes, there was an absence of fluorescence elevation indicative of a sigmoidal amplification curve.

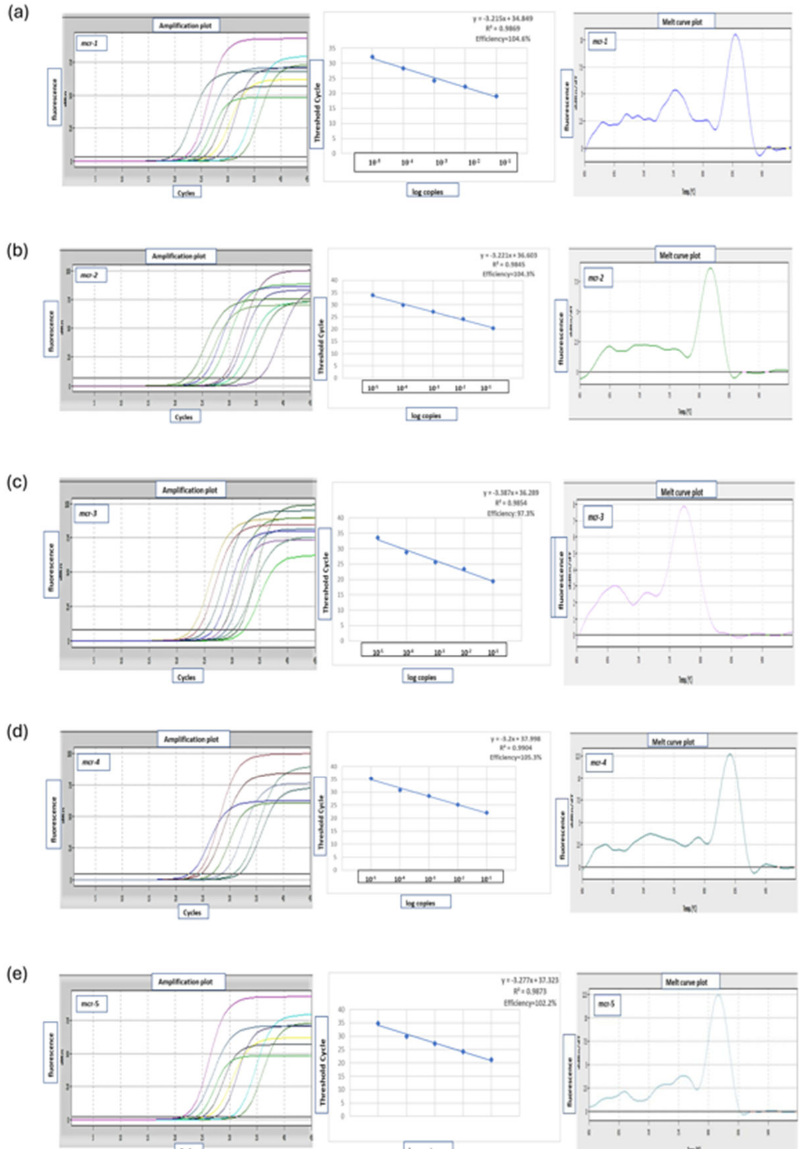

Figure 1. Real-time PCR amplification curves, standard curves, and melting curves for *mcr−1* (**a**), *mcr−2* (**b**), *mcr−3* (**c**), *mcr−4* (**d**), and *mcr−5* (**e**). The amplification curve shows important data regarding the qPCR reaction's effectiveness. A steep, exponential increase in fluorescence during the early cycles indicates efficient amplification of the target DNA. The standard curve facilitates the measurement of the target DNA quantity in unidentified samples. Through plotting known DNA concentrations against their respective threshold cycle (Ct) values, a linear correlation is established. This correlation acts as a guide to ascertain the concentration of target DNA in unknown samples, utilizing their Ct values. By employing successive dilutions of a precisely measured inoculum, real-time qPCR exhibited satisfactory efficiency, as evidenced by a standard curve showing linearity across DNA copies ranging from 34 to 19 Ct. The melting curve serves as an indicator of the overall quality of the qPCR assay. A clean, sharp melting curve with a single peak indicates robust amplification of the target sequence and reliable results.

3.3. Distribution of mcr Genes in Chicken Gut Samples

This study showed that 52.5% (63/120) of the chicken gut samples had no presence of any *mcr* gene. Conversely, the remaining 47.5% (57/120) of the samples exhibited the presence of at least one *mcr* gene out of the five variants investigated (Figure 2). The newly optimized qPCR technique worked well for identifying all the *mcr* genes in the test samples. A cut-off Ct value of 30 was determined to be ARG-detection positive. The overall

detection rates of the *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* genes were 42.5% (51/120), 2.5% (3/120), 1.7% (2/120), 5% (6/120), and 9.2% (11/120), respectively. Interestingly, different combinations of double occurrences of *mcr* genes were found in 10.8% (10/57) of the samples. The most frequent double occurrence combination was *mcr-1+mcr-5*; other combinations were *mcr-1+mcr-2*, *mcr-1+mcr-4*, and *mcr-4+mcr-5*. Three samples were detected with triple occurrences of *mcr* genes with combinations of *mcr-1+mcr-2+mcr-4*, *mcr-1+mcr-3+mcr-5*, and *mcr-1+mcr-4+mcr-5*. The highest prevalence of *mcr* genes was detected in the samples from the Dhaka district followed by those from the Gazipur district (p = 0.001).

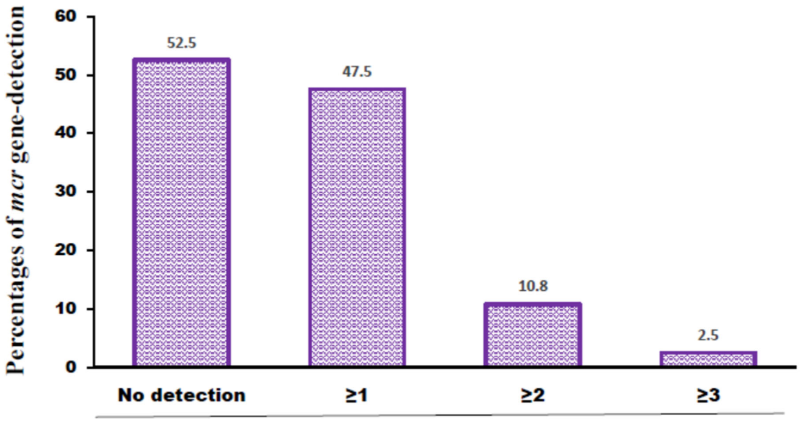

Figure 2. Cumulative distribution of *mcr* genes in chicken gut samples. The value bar shows the percentage prevalence. Only 10.8% of the tested samples carried two mobilized colistin resistance genes concurrently. Co-carriage of three or more genes was found in over 2.5% of samples.

3.4. Comparative Distribution of mcr Genes in Farm and Backyard Chicken Droppings

mcr-1 was found in 51.2% (41/80) of the commercial farm chicken droppings and 25% (10/40) in the droppings of chickens reared in households (p = 0.007). No significant difference in the prevalence of mcr-2 to mcr-5 was identified between the commercial and household chicken droppings. *mcr-2* and *mcr-3* were found in 3.8% (3/80) and 0% (0/40) of the commercial farm samples, and 0% (0/80) and 5% (2/40) of the household samples. *mcr-4* and *mcr-5* were identified in 6.25% (5/80) and 2.5% (1/40) of the farm samples, and 7.5% (6/80) and 12.5% (5/40) of the household dropping samples (Figure 3). Overall, a significantly higher number of *mcr* genes were identified in the commercial farm chicken droppings compared to the chicken droppings from households (p = 0.007).

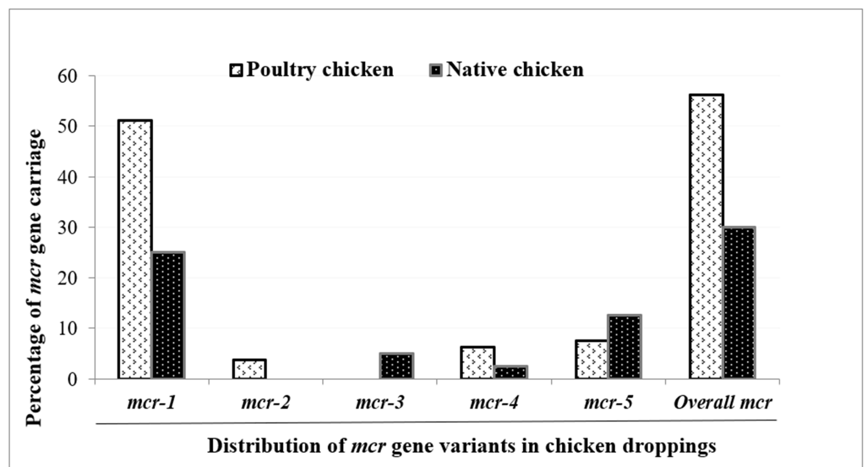

Figure 3. Comparative distribution of *mcr* genes in farm-based poultry droppings and household chicken droppings. Three *mcr* resistance genes, *mcr-1*, *mcr-2*, and *mcr-4*, were found to be higher in

commercial farming poultry droppings. Two *mcr* resistance genes, *mcr-3* and *mcr-5*, exhibited a slightly higher prevalence in household chicken gut samples. Notably, *mcr-2* was absent in native chicken droppings, while *mcr-3* was not detected in commercial poultry farms. Statistical analysis revealed statistically significantly higher presence of *mcr* genes in commercial poultry droppings ($p = 0.007$).

4. Discussion

To the best of our knowledge, this is the first study to investigate the occurrence of colistin resistance *mcr* gene variants using real-time quantitative PCR in poultry droppings in Bangladesh. We investigated the five *mcr* gene contaminants (*mcr-1* to *mcr-5*) in both commercial farm-based chicken droppings and home-based farming chicken droppings from five districts of Bangladesh. Overall, over 47% of the tested poultry droppings carried at least one type of *mcr* gene, with a higher prevalence in the samples collected around Dhaka, Bangladesh. We identified a higher mcr gene content in the commercial poultry droppings than in the household chicken droppings. The higher comparative abundance of *mcr* genes in the commercial poultry farms could be related to the greater use of colistin and other antibiotics in farm settings, which we identified in this study. The overuse and misuse of antibiotics prophylactically or therapeutically in either animal husbandry or humans were found to associated with higher accumulations of ARGs in metagenomic environments and bacterial pathogens [55]. There is always great concern about the fate and consequences of the resistance genes in poultry litter. We have no concrete evidence that the identified ARG pollution in the environment can certainly contribute to the risks of AMR acquisition in humans. However, ARGs may create selection pressure for evolving resistant pathogens [56,57]. *mcr* genes are plasmid-mediated, having a rapidly emerging capacity to be disseminated from one bacterium to another by HGT [40,41]. Using poultry litter as manure in agricultural lands and natural rainwater can disseminate ARGs further into other health domains. Humans can be exposed to ARGs or ARG-carrying bacteria by direct contact with a polluted environment, fecal waste, food, and/or drinking water [58,59]. Consequently, the potential impact of ARGs in the food value chain and environment on AMR emergence needs to be evaluated alongside possible control practices in national and global policy documents. This is already happening, with many countries banning the use of colistin as a growth promoter in animal feeds [22,35,36,60].

More than 10% of the tested samples had two or more colistin resistance genes present. To the best of our knowledge, this research is also the first to report the identification of *mcr-1* to *mcr-5* in Bangladesh. Some earlier studies have reported *mcr-1*, *mcr-2*, and *mcr-3* genes in poultry, the environment, and clinical bacteria [22,52,61–64]; however, reports of *mcr-4* and *mcr-5* had not yet been seen in Bangladesh. We also believe this study is unique in Bangladesh because we reported *mcr* genes using culture-independent real-time qPCR analysis. We optimized a Sybr Green-based qPCR and melting plot analysis to quickly and accurately find and measure the different ARGs in chicken feces. qPCR can identify ARGs rapidly, precisely, and reliably in samples where conventional microbiology takes a much longer time. This is important in countries such as Bangladesh. With culture-based microbiology, it is sometimes critical or impossible to grow injured or fastidious bacteria [65]. Because of the higher sensitivity of qPCR, the *mcr* gene detection rate appeared to be much higher than that reported previously in research using conventional PCR detection [22,52,61,64,66]. Similarly, the prevalence of *mcr* genes in India, Pakistan, and Nepal was reported to be between 9.2% and 27.6% according to conventional PCR [67–69]. As such, culture-independent molecular analyses can be helpful for the surveillance of antibiotic resistance profiles in environmental and clinical samples where conventional microbiology is challenging, and where there are key concerns including increased resistance to antibiotics in the 'Reserve' group. We will continue to evaluate this new technique and seek to rapidly introduce it into microbiological laboratories in Bangladesh as we seek to improve the communication of resistance patterns and their implications. Strengthening the monitoring of antibiotic usage can be achieved through enforced regulations, the

implementation of antibiotic stewardship programs, and transparent reporting systems. Promoting environmentally friendly commercial poultry farms involves encouraging sustainable practices and offering financial incentives for eco-friendly operations. Education and training for farmers on responsible antibiotic use and environmental impacts are crucial. Collaborative efforts among stakeholders will enhance knowledge sharing and foster an improvement in sustainability and responsibility in poultry farming. To educate farmers on these issues, awareness-building programs that increase biosecurity, vaccination, and antimicrobial stewardship must be created. Most of all, the government should play a key role in this regard while coordinating the activities of donor agencies, law-enforcing bodies, pharmaceutical industries, professionals, and research institutes. It is mandatory to monitor antibiotic manufacturing plants, drug licensing bodies, and poultry farms to confirm the adherence to guidelines provided by the Government to supervise illegal antibiotic production, selling, importing, and usage. Where applicable, strict penalties in the form of monetary fines, jail time, and cancelation of licenses and registrations should be employed. These guidelines could be aligned with the goals of the National Action Plan of Bangladesh to reduce AMR [70].

This study had several limitations, including its cross-sectional design and lack of follow-up due to resource constraints. The collected antimicrobial usage history in this study was self-reported by the chicken farm owners and has not been validated by other independent investigations. This study did not investigate the acquisition of ARGs in bacterial communities through microbiological methods. Future research should focus on determining the presence of ARGs in bacteria and their phenotypic antimicrobial susceptibilities. This study covered five variants of the *mcr* (*mcr-1* to *mcr-5*) genes; other gene variants such as *mcr-6* to *mcr-10* were not explored.

5. Conclusions

The existence of *mcr* genes in poultry feces in Bangladesh emphasizes the importance of proper poultry waste disposal and good hygiene practices among people who work closely with livestock and poultry meats. The Sybr Green-based quantitative polymerase chain reaction method appeared to be a rapid, accurate, and highly sensitive screening technique for the *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* genes from the culture-independent chicken gut contents. Validated RT-qPCR could be employed as a sensitive and reliable technique for designing any AMR surveillance method in livestock, the environment, and clinical settings. The potential impacts of ARGs in the food value chain on AMR emergence should be considered in national and global policy documents. An integrated and combined approach to the One Health concept should be applied in all domains to understand and control the environment's role in the evolution and transmission of AMR. Further extensive research is imperative to discover new methods and strategies to neutralize genomic and metagenomic ARG contaminants in different One Health sectors. This is particularly important in countries such as Bangladesh, with critical concerns regarding AMR, especially among antibiotics in the 'watch' and 'reserve' groups.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/sci6040076/s1, Figure S1: Sampling spot map.

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Institutional Review Board Statement: This study was an extension of an earlier research project approved by the Ethics and Research Review Committee of the Jahangirnagar University Faculty of Biological Sciences (approval number: BBEC, JU/M 2017 12(4), approval date: 27 December 2017). The ethical committee has waived the necessity for new approval.

Informed Consent Statement: The study followed all the standard ethical guidelines and regulations for environmental samples. Each poultry farm/owner was informed about our study purposes and verbal consent was taken before sample collection. Personal and farm identities were kept anonymous. A sample identification code was assigned for each sample collected to protect personal and business identities.

Data Availability Statement: Research data will be available upon request to the corresponding author.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

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