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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

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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.

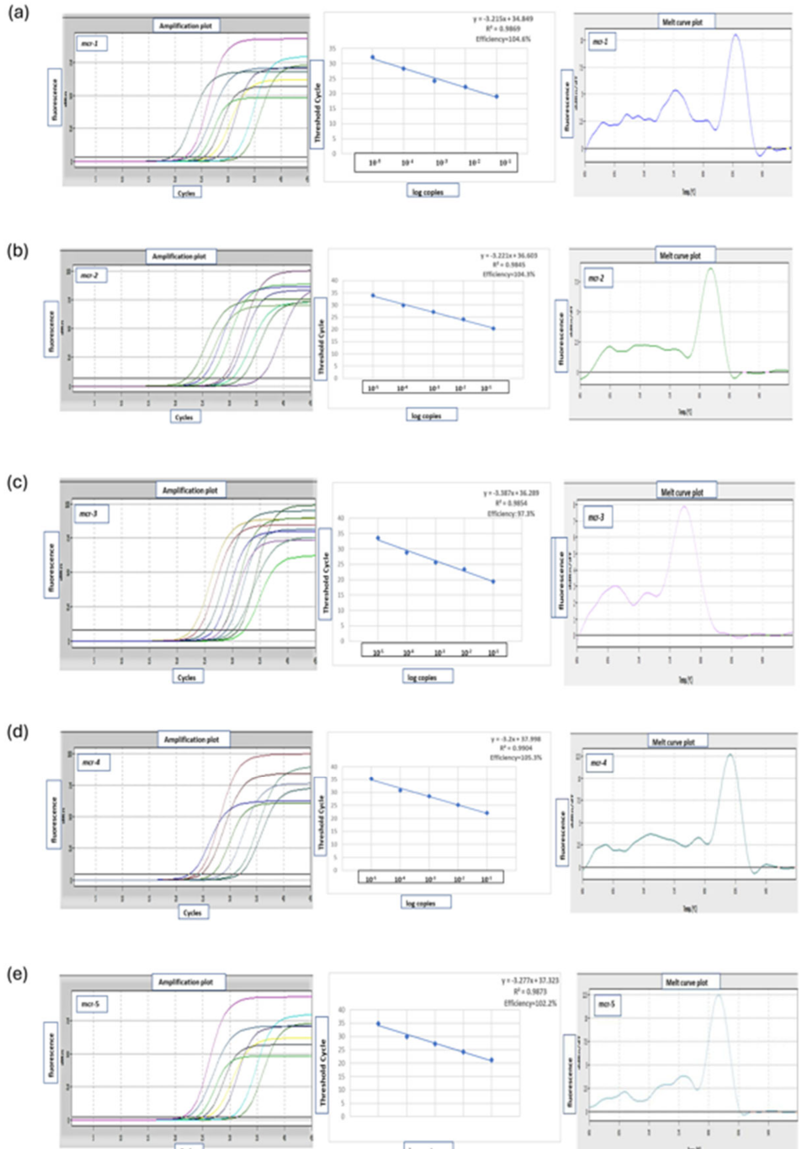

Figure 1. Real-time PCR amplification curves, standard curves, and melting curves for *mcr−1* (**a**), *mcr−2* (**b**), *mcr−3* (**c**), *mcr−4* (**d**), and *mcr−5* (**e**). The amplification curve shows important data regarding the qPCR reaction's effectiveness. A steep, exponential increase in fluorescence 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).

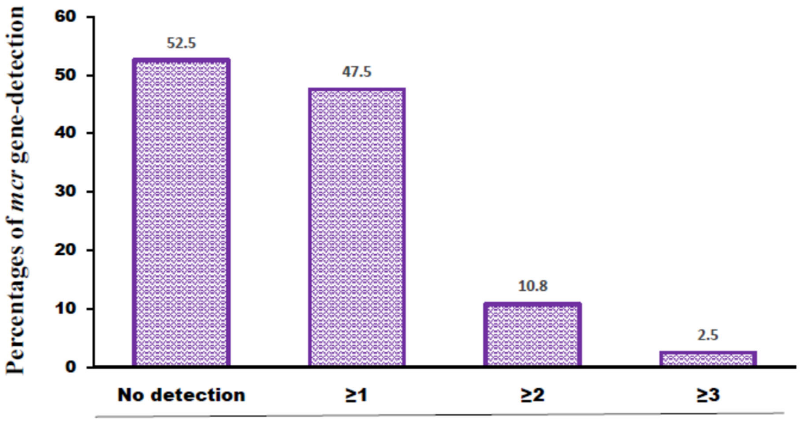

Figure 2. Cumulative distribution of *mcr* genes in chicken gut samples. The value bar shows the percentage prevalence. Only 10.8% of the tested samples carried two mobilized colistin resistance genes concurrently. Co-carriage of three or more genes was found in over 2.5% of samples.

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).

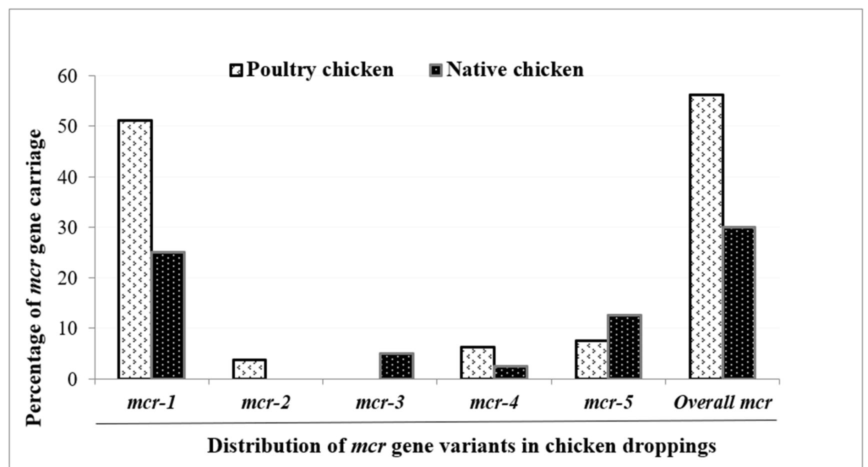

Figure 3. Comparative distribution of *mcr* genes in farm-based poultry droppings and household chicken droppings. Three *mcr* resistance genes, *mcr-1*, *mcr-2*, and *mcr-4*, were found to be higher in

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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