

## Original Article

# Screening and Identification of Chromium Resistant Bacteria from Poultry Excreta

Md. Abu Sayem Khan<sup>1</sup>, Md Muzahidul Islam Badhon<sup>1</sup>, *Supantha Rivu*<sup>1</sup>, Sabita Rezwana Rahman<sup>1\*</sup>

<sup>1</sup>Department of Microbiology, University of Dhaka. Dhaka-1000, Bangladesh

The occurrence of heavy metal-resistant bacteria in the environment acts as an indicator of heavy metal pollution. The impact of heavy metal contamination in co-selection and proliferation of antimicrobial-resistant bacteria is well-documented in existing literature. The present study aims to determine the occurrence of chromium-resistant bacteria (CRB) in poultry feces and to characterize their antimicrobial resistance and biofilm formation ability. 28 CRBs were isolated by inoculating samples on chromium-amended media. 6 of 28 isolates were resistant to a maximum of 1000 ppm chromium. Isolated CRBs also showed varying degrees of resistance to other heavy metals, including Cd, Ni, and Hg. Biofilm formation was observed in 67.8% isolates, of which 60.7% and 7.1% were weak and moderate biofilm former, respectively. All the isolates exhibited sensitivity to Gentamicin, Chloramphenicol, Imipenem, and Amikacin. Only one isolate was multi-drug resistant. In addition, all the isolates possessed chromate reductase gene that confirmed their chromium-reducing activity. Two isolates were identified as *Bacillus altitudinis* and *Brevibacillus parabrevis* on the basis of 16s rRNA analysis. Further estimation of chromium-reduction capacity and whole genome analysis will reveal their bioremediation potential.

**Keywords:** Chromium, Poultry, Excreta

## Introduction

Discharge of chromium-containing solid or liquid wastes from different industries, especially leather tanning, textile, electroplating, and others, causes chromium pollution in the environment<sup>1,2</sup>. Among the different oxidation states, Cr(III) and Cr(VI) are the most abundant chromium compounds in the natural environment, with different physicochemical characteristics and biological roles<sup>3</sup>. Among these two states, trivalent chromium is less stable and, relatively insoluble and immobile than its hexavalent form<sup>4</sup>. The United States Environmental Protection Agency enlisted hexavalent chromium as Group “A” human carcinogen because of its strong oxidation potential, membrane permeability, and water solubility<sup>5</sup>.

Skin trimmings, keratin wastes, chrome shaving wastes, and buffing wastes are the main types of solid wastes produced in the tannery industry<sup>6,7</sup>. Discharge of chromium containing liquid and solid wastes into canals and rivers results in aquatic and terrestrial pollution<sup>8</sup>. Earlier studies reported that the solid wastes were being processed by several big and small mills into protein concentrate for manufacturing poultry feed in Bangladesh<sup>9,10</sup>. One study reported a high concentration of chromium in chicken, which might be attributed to the contamination of feed with leather wastes<sup>11</sup>. Through these feeds, accumulation of chromium occurs in chicken body parts and facilitates its entry into the human food chain. Heavy metals can cause some toxicity in human, including hepatotoxicity, neurotoxicity, and teratogenicity<sup>12,13</sup>.

Hexavalent chromium is known also to cause several health complications such as, placental accumulation, impaired fetal growth, skin allergies, vomiting, diarrhea, and others<sup>14</sup>.

Bacteria adopt numerous protection mechanisms including active transport of metal ions, extracellular and intracellular sequestration, reduction of metal ions and others, that prolong their survival at higher concentration of heavy metal<sup>15</sup>. The ability to reduce chromium with chromate reductase enzymes has been identified in some CRB<sup>16</sup>. Bacteria resistant to a particular heavy metal might also resist other heavy metals. In addition, chromium resistance is often linked to antibiotic resistance<sup>17–19</sup>. Though the use of chromium contaminated tannery wastes in poultry feed and its presence in chicken organs had been reported, no studies on the presence of CRB, their contributions in multiple heavy metals and antibiotic resistance, and bioremediation potential have been made so far from poultry environment in Bangladesh. In this perspective, our objective was to demonstrate the occurrence of chromium resistant bacteria in collected poultry excreta samples from major poultry producing areas surrounding Dhaka city and characterize them in terms of their antimicrobial resistance and biofilm formation.

## Materials and Methods

### Sample collection and processing

20 fresh poultry dropping samples were collected from different commercial poultry farms in Dhamrai (Dhaka) and Sreepur

\*Corresponding author:

Sabita Rezwana Rahman, Department of Microbiology, University of Dhaka. Dhaka-1000, Bangladesh, email: [sabita\\_rahman@du.ac.bd](mailto:sabita_rahman@du.ac.bd)

(Gazipur) in November 2021. Samples were transferred to the laboratory and processed within 6 hours of collection. Samples were diluted using sterile normal saline up to  $10^{-8}$ , and then, 100 $\mu$ l of each dilution was spread on Luria-Bertani agar plate supplemented with 100 mg/L potassium dichromate ( $K_2Cr_2O_7$ ). Inoculated plates were incubated at 37°C for 48-72 hours. Selected single colonies were streaked onto Nutrient Agar plates to obtain pure culture of isolates.

#### *Tolerance to different heavy metals*

The Minimal Inhibitory Concentrations (MIC) of different heavy metals (Chromium, Cadmium, Mercury, and Nickel) for each isolate were determined by agar plate dilution. Isolates were inoculated on LB agar supplemented with different concentrations of Cr (VI) (100-1500 mg/l), Cd (II) (25-200 mg/l), Hg (II) (25-100 mg/l), and Ni (II) (100-1000 mg/l) and incubated at 37°C for 48-72 hours. The lowest concentration of metal inhibiting bacteria's visible growth was defined as MIC.

#### *Biofilm Formation Assay*

Qualitative and quantitative characterization of bacterial biofilm forming ability was done using crystal violet microtiter plate assay. 50 $\mu$ l of freshly grown diluted (1:100) bacterial cultures were inoculated in triplicate on 96 well flat bottom microtiter plate containing 100 $\mu$ l LB broth. Following incubation at 37°C for 24 hours, the plates were washed with sterile water to remove planktonic cells. Then, the adherent cells were stained with 200  $\mu$ l of 0.1% crystal violet for 30 minutes. The plates were rinsed again with deionized water, and allowed to dry. Finally, 200 $\mu$ l of 95% ethanol was added to all wells to solubilize the crystal violet and the absorbance was taken at 492nm. The extent of biofilm-formation was interpreted in accordance with Stepanovic criteria<sup>20</sup>. The Stepanovic criteria are given below: ( $OD_d - OD_c =$  No biofilm former,  $OD_c < OD_d - 2 \times OD_c =$  Weak biofilm former,  $2 \times OD_c < OD_d - 4 \times OD_c =$  Moderate biofilm former,  $4 \times OD_c < OD_d =$  Strong biofilm former)

#### *Antibiotic Susceptibility test*

Kirby-Bauer disc diffusion method was followed to determine the antibiotic susceptibility of the isolates<sup>21</sup>. The following antibiotics were used: Gentamicin (10 $\mu$ g), Tetracycline (10 $\mu$ g), Chloramphenicol (30 $\mu$ g), Vancomycin (30 $\mu$ g), Imipenem (10 $\mu$ g), Cefepime (30 $\mu$ g), Erythromycin (15 $\mu$ g), Ciprofloxacin (5 $\mu$ g), Amikacin (30 $\mu$ g), Amoxicillin (30 $\mu$ g). Isolates were inoculated on Mueller Hinton broth and incubated at 37°C until 0.5 McFarland standard ( $1.5 \times 10^8$  cells/ml) was obtained. Then, cultures were streaked on the Mueller Hinton agar using a sterile cotton swab to get a homogenized bacterial lawn. The antibiotic discs were placed on the surface of the inoculated agar plate. Diameters of zones of inhibition were measured after overnight incubation at 37°C. Interpretation of the diameter of the zone of inhibition was done (mm) according to CLSI guidelines<sup>22</sup>.

#### *DNA extraction and amplification of chromate reductase gene*

The Boil DNA extraction protocol was followed for extraction of chromosomal DNA<sup>23</sup>. Then, polymerase chain reaction (PCR) was carried out to detect chromate reductase gene. The primer sequences, amplicon size and PCR conditions are listed in Table 1. The total volume of each PCR reaction was 25 $\mu$ l comprising 12.5 $\mu$ l 2X Mastermix (Promega, USA), 1 $\mu$ l forward and reverse primer each, 8.5 $\mu$ l nuclease-free water, and 2 $\mu$ l template DNA. The amplified PCR products were separated on 1.2% agarose gel in Tris-Borate-EDTA (TBE) buffer stained with Ethidium bromide. (0.5 mg/mL).

#### *Molecular identification of chromium reducing bacteria*

The 16s rRNA gene was amplified by PCR using universal primers. Then, Wizard® SV Gel and PCR Clean-Up System was used to purify PCR products, followed by sequencing using the Sanger method. BioEdit was employed for editing raw sequences. The fasta file was blast searched to identify matches with available characterized sequences. Then, *phylogenetic tree* construction based on *neighbor joining* method was done using *MEGA 11* software, with 1000 bootstrap values.

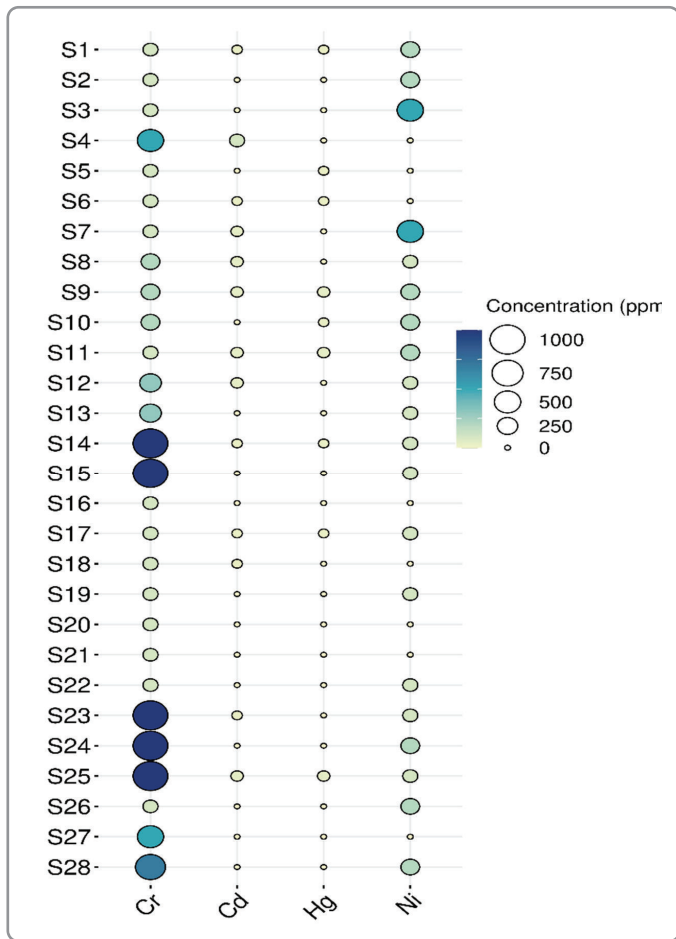
**Table 1:** Primers used to amplify chromate reductase and 16s rRNA

Serial No	Target gene	Primers	Primer Sequence	Amplification conditions	Amplicon Size	Characteristics
1	16S	F (5'→3')	AGAGTTTGATC CTGGCTCAG	Denaturation: 94°C, 60s Annealing: 49.5°C,	1465bp	Structural component of ribosome
		R (5→3')	CTACGGCTA CCTTGTTACGA	60sExtension: 72°C, 90s		
2	Chromate reductase	F (5'→3')	TCACGCCGGAAT	Denaturation: 94°C, 60s	268bp	Chromium tolerance
		R (5'→3')	CGTACCCTGATCA ATCACTT	Annealing: 49.5°C, 60s Extension: 72°C, 90s		associated genes

**Results and discussion**

Twenty Eight morphologically different bacterial isolates were selected based on their ability to grow on chromium-supplemented media. These isolates were further inoculated on LB agar to determine their tolerance to varying concentrations of hexavalent chromium (100-1500 mg/l). 6 out of 28 (21.4%) isolates showed resistance to 1000 mg/l Cr (VI) and 8 isolates had MIC between 300 to 700 mg/l hexavalent chromium. Earlier studies also reported the isolation of CRBs with the ability to tolerate hexavalent chromium at concentrations of 500–2,500 mg/l<sup>8,24</sup>. Bacteria resistant to one heavy metal might also develop resistance to

other heavy metals<sup>25</sup>. Isolated CRBs showed varying extents of tolerance to different concentrations of heavy metals including cadmium, nickel and mercury. Only one isolate exhibited resistance to a maximum of 100 ppm Cd (II). The MIC of Cd (II) for the remaining isolates was d 50 mg/l. Maximum isolates (67%) had MIC d 25 mg/l for Hg (II). Isolates showed greater tolerance to nickel than mercury and cadmium. 2 isolates had MIC of 500 ppm Ni (II). The MIC of Ni (II) for the remaining isolates was determined to be d 5 0 mg/l (Figure 1). These findings are in agreement with previous studies that also reported multiple heavy metal resistance characteristics of CRB<sup>9,26–29</sup>.



**Fig. 1:** Heavy metal tolerance of each isolate

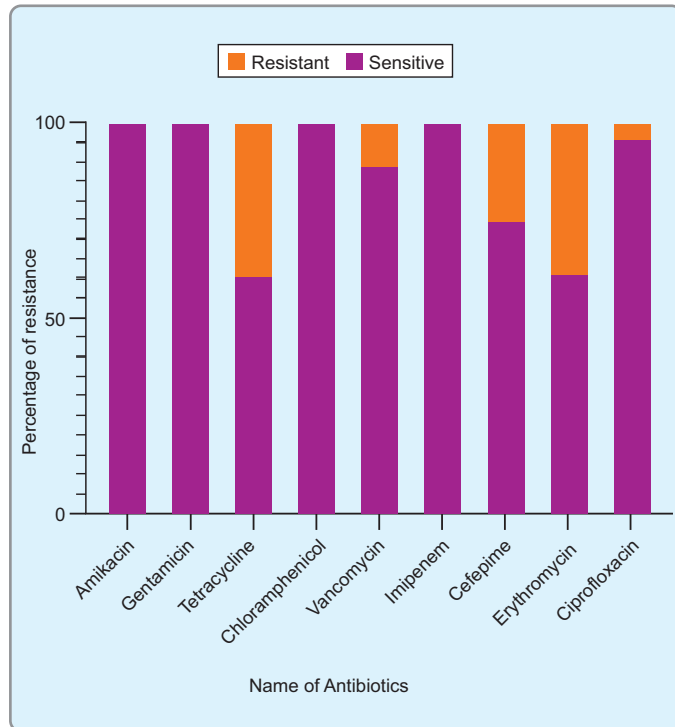
Biofilm formation protects bacteria from heavy metal toxicity by sequestration or complexation of heavy metals and limiting their diffusion into biofilm<sup>30,31</sup>. In this study, 67.86% (n=19) isolates produced biofilm in microtiter plate assay, being distributed into the following categories: 60.72% (n=17) formed weak biofilm, 7.14% (n=2) had moderate biofilm, while 32.14% (n=9) showed no biofilm formation (Table 2). Biofilm formation by chromium-resistant *Stenotrophomonas maltophilia*, *Aspergillus niger*, *Staphylococcus arlettae* with or without chromium stressed conditions had been described in other studies<sup>3,32,33</sup>.

An expanding body of studies suggested that heavy metal ions decrease antimicrobial susceptibility by co-regulation of antimicrobial resistance (AMR) genes<sup>34,35</sup>. In the contrary, a decrease in antibiotic resistance by increasing heavy metal concentrations had also been reported<sup>36–38</sup>. Here, all of the isolates (100%) were sensitive to the action of gentamicin, amikacin, imipenem, and chloramphenicol, followed by ciprofloxacin (96.5%), vancomycin (89%), and cefepime (75%). 39% isolates (n=11) exhibited resistance to tetracycline and erythromycin (Figure 2). The isolates that showed resistance to two or more different classes of antibiotics were 6 and 1 (Multidrug-resistant), respectively. These isolates also showed varying degree of resistance to different heavy metals as described earlier, suggesting the co-occurrence of antimicrobial and heavy metal-resistance. Similarly, a study by Roopali et al., observed that most chromium-resistant bacteria isolated from fly ash

**Table 2:** Biofilm formation activity of isolates

Biofilm Category	OD range	Isolate ID	No of Isolates (Percentage)
No	OD d'' ODc (0.07)	S8, S10, S12, S18, S19, S20, S25, S27, S28	9 (32.14%)
Weak	ODc < OD d'' 2ODc	S2, S3, S4, S5, S6, S7, S9, S11, S13, S14, S15, S16, S17, S21, S23, S22, S26	17 (60.72%)
Moderate	2ODc < OD d 4ODc	S1, S24	2 (7.14%)

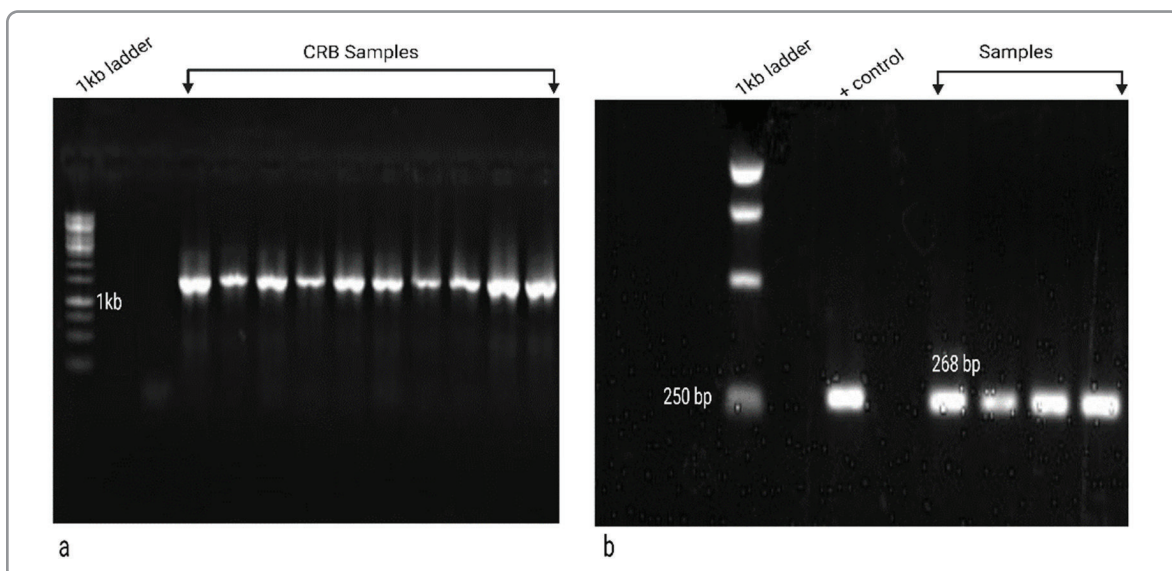
samples were resistant to one or more antibiotics<sup>29</sup>. Another study reported the isolation of *Stenotrophomonas maltophilia* NA2 with the MIC of 7400mg/L Cr (VI) that was resistant to different classes of antibiotics, notably meropenem, aztreonam, and amikacin<sup>3</sup>.



**Fig. 2:** Graphical representation of the antimicrobial sensitivity of Chromium Resistant Bacteria (CRB)

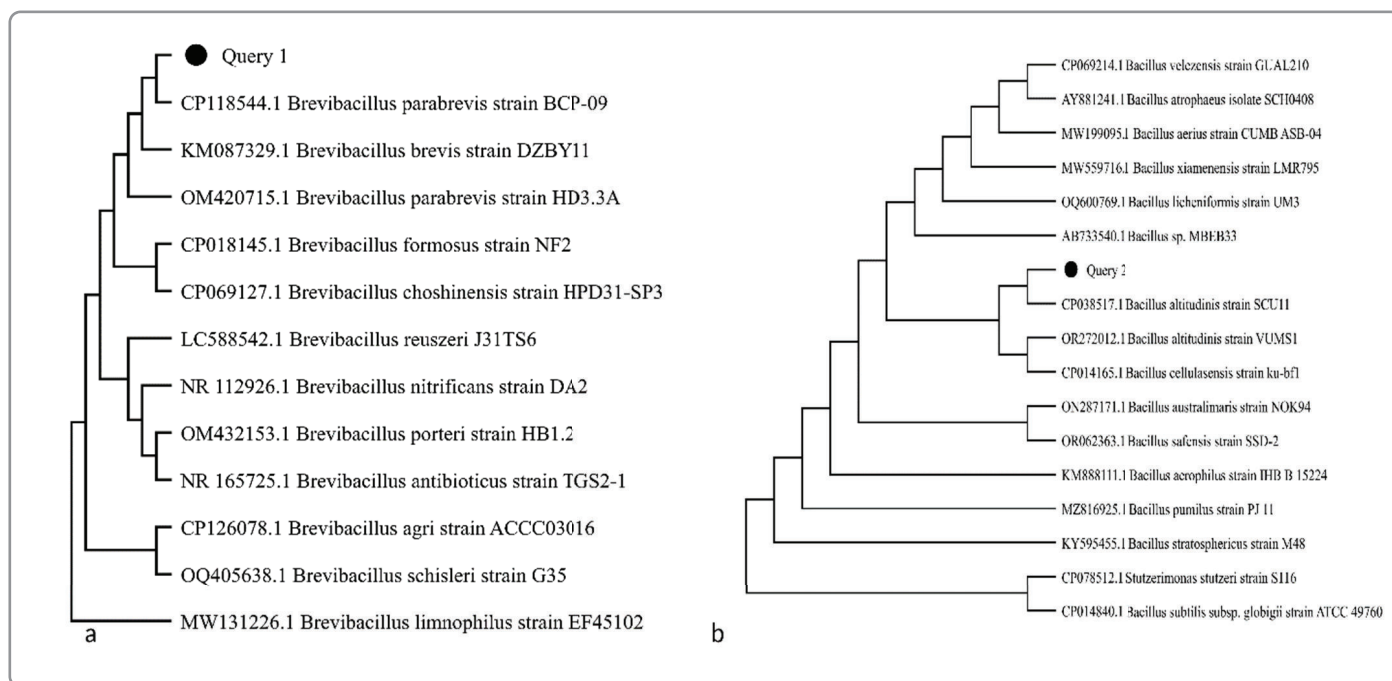
Chromium reductase, which catalyzes the reduction of Cr(VI) to Cr(III), can be found in chromosomal DNA or plasmid or both<sup>39</sup>. The involvement of the *ChrR* gene in bacterial chromate resistance mechanism had been well-established<sup>40</sup>. Here, the amplified *ChrR* gene yielded a 268bp band compared to the DNA ladder, confirming the presence of chromate reductase gene in the chromosomal DNA of all isolates, and thus, reaffirming their chromate-reducing property (Figure 3). Several studies partially amplified this gene to confirm chromate reductase activities of isolates<sup>1,3,41,42</sup>.

The genotypic identification of two isolates that showed antibiotic and metal resistance as well as biofilm formation, was done using 16s rRNA sequencing. BLASTn analysis identified isolate S-2 (Query 1) and S-24 (Query 2) as *Brevibacillus parabrevis* (Accession: CP118544.1; identity: 99.67%, Coverage: 100%) and *Bacillus altitudinis* (Accession: CP038517.1; identity and Coverage: 100%), respectively. Phylogenetic trees were constructed by the neighbor-joining method with 1000 bootstrap replicates (Figure 4). Numerous studies described the heavy metal resistance characteristics of *Brevibacillus parabrevis* and *Bacillus altitudinis* and their subsequent utilization in heavy metal bioremediation and hydrocarbon degradation. Wani et al. achieved a significant reduction of Cr(VI) by *Brevibacillus parabrevis* OZF 5<sup>16</sup>. Utilization of lead-resistant *Brevibacillus parabrevis* AMB-CD-2 resulted in a substantial decrease (58.75%) of lead compared to control<sup>43</sup>. Harsonowat et al., reported the effective and superior multi-metal degrading capacity (91.30% for Hg, 98.07% for Cu, and 54.36%) of two *B. altitudinis* isolates<sup>44</sup>. *B. altitudinis* JUGS2C showed tolerance to a maximum of 175 mg/l Cr(VI) and reduced 40% of 25 mg/L Cr(VI) at optimized conditions<sup>45</sup>.



**Fig. 3:** PCR product for 16s rRNA (a) chromate reductase (b) gene on 1.2% agarose gel





**Fig. 4:** 16s rRNA based phylogenetic tree of S-2 (a) and S-24 (b) compared with closely related organisms

**Conclusion**

In this study, 28 CRBs were isolated from poultry feces that showed resistance to different concentrations of heavy metals, including cadmium, nickel, and mercury. Most isolates were biofilm former and exhibited resistance to one or more tested antibiotics. Two isolates were identified as *Bacillus altitudinis* and *Brevibacillus parabrevis* by 16s rRNA sequencing and analysis. More studies on the measurement of chromium-reducing activity, molecular analysis on the co-occurrence of antibiotic and heavy metal resistance genes, and transfer of metal resistance determinants are needed to depict the comprehensive scenario of chromium resistance among microorganisms prevailing in poultry environments.

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**Conflict of Interest**

The authors declare no conflict of interest.

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