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1 **Antimicrobial Resistance Profile and *mcr-1* Gene Detection in *Salmonella* Isolates from**
2 **Poultry in Bangladesh: Molecular and Bioinformatics Characterization**
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32 **Running title:**
33 ***mcr-1* gene Detection in Salmonella in Poultry**
34

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35 **Antimicrobial Resistance Profile and *mcr-1* Gene Detection in *Salmonella* Isolates from**
36 **Poultry in Bangladesh: Molecular and Bioinformatics Characterization**
37
38
39
40 **Abstract**

41 Antimicrobial resistance gene *mcr-1* has been disseminated globally since its first discovery in
42 Southern China in late 2015. However, the *mcr*-1 gene had not been identified previously in
43 *Salmonella* isolates from poultry in Bangladesh. Here, we aimed to explore antimicrobial
44 resistance gene *mcr-1* in *Salmonella* isolates. Eighty two *Salmonella* isolates were isolated and
45 characterized from suspected poultry specimens received from different zones of the country. A
46 phenotypic disc diffusion assay with 15 antimicrobial agents was performed following CLSI
47 standard. The disk diffusion assay showed that, all of the isolates presented high resistance to
48 colistin (92.68%), oxytetracycline (86.59%), co-trimoxazole (76.83%), ciprofloxacin (73.17%)
49 and enrofloxacin (65.85%). Further, randomly selected 10 *Salmonella* isolates were analyzed by
50 polymerase chain reaction (PCR) targeting genus-specific *invA* and antimicrobial (colistin)
51 resistance *mcr*-1 genes. Five were confirmed for the presence of the *mcr-1* gene belonging to
52 *Salmonella* spp. Further, sequencing followed by phylogenetic analysis revealed divergent
53 evolutionary relation between the LptA and MCR proteins rendering them resistant to colistin.
54 Three-dimensional homology structures of MCR-1 proteins were constructed and verified using
55 different bioinformatics tools. Moreover, molecular docking interactions suggested that, MCR-1
56 and LptA share a similar substrate binding cavity which could be validated for the functional
57 analysis. The results represent here is the first molecular and *in silico* analysis of colistin
58 resistance *mcr-1* gene of *Salmonella* in poultry in Bangladesh, which may emphasize the
59 importance of the study on antibiotic resistance genes requiring for national monitoring and
60 strategic surveillance in the country.
61

62 **Keywords:** Antimicrobial resistance, *mcr-1* gene, *Salmonella*, Poultry, Bangladesh
63

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64

65 **Introduction**

66 Poultry (mainly broiler and layer) farming is an important avenue in fostering agricultural
67 growth and has becoming a major contributing sector for potential income generation and
68 poverty alleviation in Bangladesh (1, 2). Eggs and poultry meats are most acceptable and largely
69 consumed animal products to meet dietary nutritional requirements throughout the country (3,
70 4). In spite of significant improvement, there is a potential threat of diseases due to bacterial
71 infections that can result a huge economic loss in this sub-sector (5, 6). Among them,
72 infections with *Salmonella* spp. are the most commonly reported problem in poultry that cause
73 food borne illness to human and remain as persistent threat to both human and animal health (6–
74 8) . Globally, 94 million human affected cases were estimated due to *Salmonella* spp. leading to
75 155,000 deaths every year (9, 10).

76

77 Salmonellosis is endemic in nature causing morbidity and mortality in poultry. It is very
78 significant by virtue of the fact that, it can be transmitted vertically from parent to offspring; this
79 makes its control a challenge. Although vaccination and good hygiene practices are most
80 effective ways to prevent salmonellosis (11), antibiotics are extensively using either as a growth
81 promoter or prophylaxis and therapeutics in poultry industry of Bangladesh (12, 13). Indeed, the
82 widespread misapplication and nonjudicious use of antimicrobial drugs in poultry settings,
83 culminating the development of antimicrobial resistant pathogens like *Salmonella* (Gyles, 2008,
84 Cantas et al., 2013; Antunes et al., 2016).

85

86 Antimicrobial resistant *Salmonella* of poultry can harbor as a major risk and vehicle for
87 dissemination of these pathogens to humans (16, 17). Standard culture, biochemical and
88 serological methods are usually employed for isolation and identification of *Salmonella* species.
89 However, the *invA* encoding invasion gene, commonly involved in bacterial virulence, is
90 accountable and routinely used for the detection of *Salmonella* spp. (18). Moreover, the *invA*
91 sequences are distinctive to the genus *Salmonella* and diagnosed by polymerase chain reaction
92 (PCR) is a preferred diagnostic method due to its reliable sensitivity, specificity and detection
93 speed (19, 20).

94

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Of note, poultry is usually incriminated in outbreaks of human Salmonellosis. Therefore, the detection of *Salmonella* species in poultry production chain particularly at the farm level is an issue of great concern. Furthermore, the resistance of some *Salmonella* serotype to multiple antibiotics (15) makes the study on antibiotic susceptibility profile and its antimicrobial resistance gene, a great priority (21). So far, *mcr-1* positive *Enterobacteriaceae* (MCRPE) has been found in animal, food, human and environment in over 25 countries across 4 continents (22–25). As far as literature mining is concerned from PubMed search regarding poultry in Bangladesh, no data was found on the molecular characterization and antimicrobial resistance gene detection in *Salmonella*.. Therefore, the present study has undertaken to detect colistin resistance *mcr-1* gene for the first time in *Salmonella* isolates and its associated drug resistance pattern of commonly used antibiotics.

In this study, morphological and biochemical techniques were generated and phenotypic and genotypic characteristics of isolates were explored using antimicrobial susceptibility testing, PCR, nucleotide analysis, bioinformatics and structural modeling of bacterial genetics. The phylogenetic relationships between local isolates and published data sets from different corner of the world were analyzed. Additionally, molecular docking of phosphatidylethanolamine substrate with MCR-1 and LptA were investigated. Focus has been given on antimicrobial resistance *mcr-1* gene involved in multidrug leading to colistin resistance.

Materials and methods

Ethical standards

The research has been conducted in accordance with the Institutional Ethics Committee of Kazi Farms Group, Dhaka and Sylhet Agricultural University (SAU), Sylhet, Bangladesh.

Study area and sampling

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126 The study was conducted from January to June 2019 at popular poultry zones of Bangladesh:
127 Gazipur, Narsingdi, Tangail and Brahmanbaria (**Figure 1**). Samples of dead and sick birds were
128 collected and transferred to the customer service lab at Kazi Farms Group, Gazipur, Bangladesh.
129 Postmortem was conducted immediate after receiving samples with their anamnesis and clinical
130 information in accordance with the standard guidelines by a veterinarian. During postmortem,
131 liver and intestinal samples were collected and immediately sent for further analysis. For sick
132 live birds, blood was collected for serum separation before the postmortem examination.
133

134 **Isolation and biochemical identification of bacterial isolates**

135 Before isolation of *Salmonella* spp, samples were initially screened out by Rapid Serum Plate
136 Agglutination Test (RSPAT; ID Vet, France) followed by clinical and postmortem findings.
137 Liver and intestinal samples from 100 suspected samples were subjected to a pre-enrichment step
138 by combining with 225 mL Buffered Peptone Water (BPW) in a ratio of 10 fold dilutions and
139 incubated at 37°C for 24 hours (h). For *Salmonella* specific pre-enrichment, culture were further
140 transferred to Modified Semi-solid Rappaport Vassiliadis (MSRV; Hi Media, India) and
141 Tetrathionate Broth (TTB; Hi Media, India) consecutively and incubated at 42°C for 24 h.
142 Following enrichment, a loop of enriched broth was initially streaked on Xylose-Lysine-
143 Desoxycholate (XLD; Hi Media, India) agar and colonies (single pinkish) were streaked on
144 *Salmonella-Shigella* (SS) agar, incubated at 37°C for 24 h. Salmonella colonies were identified
145 by physical and biochemical properties using Gram's stain, catalase and indole tests as
146 previously described (Dashti et al., 2009; Sobur et al., 2019). For further confirmation, 4-6
147 suspected *Salmonella* colonies from each samples were tested biochemically by dilution
148 streaking and stab onto Triple Sugar Iron (TSI) agar (Merck, Germany) and incubation at 37°C
149 for 16-24 h (28, 29).
150

151 **Antimicrobial susceptibility testing**

152 *Salmonella* isolates were examined for phenotypic antibiogram using 15 antimicrobial agents by
153 Kirby–Bauer disk diffusion method as previously described (30). In a brief, isolates were grown
154 on Mueller-Hinton (MH) agar (Hi Media, India) and incubated for 16-18 h at 37°C. McFarland
155 0.5 standards were maintained for culture suspension of individual isolate. Discs were placed on
156 the agar surface using a sterile forceps and incubated at 37°C for 18 hours. The tested results

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157 were interpreted by measuring the zones of inhibition and scored as sensitive, intermediate and
158 resistant according to the CLSI (CLSI, 2019) (**Table1**). Isolates that were found resistant against
159 at least 3 classes of antibiotics considered as multidrug resistance (MDR) *Salmonella* spp (**Table**
160 **2**) (32).
161
162

163 **Extraction of Bacterial genomic DNA**

164 Among 82 *Salmonella* isolates, 10 isolates were randomly subjected to molecular
165 characterization for the identification of resistance genes. For this, bacterial DNA was extracted
166 by boiling-centrifugation method as described earlier (27, 33). In a brief, a loop full of overnight
167 cultured bacterial suspension was transferred into 1.5 mL microcentrifuge tube and centrifuged
168 at 13,000 rpm for 1 min. The supernatant was discarded and 1 mL of sterile ultrapure water was
169 added and vortexed. The suspension was heated at 100°C for 8~10 min in a heating block and
170 then immediately cooled on ice for 5 min. Cell debris from the cell lysates were pelleted by
171 centrifugation at 13,000 rpm for 1 min and remaining supernatant was used as DNA templates
172 for PCR assays.
173

174 **Polymerase Chain Reaction (PCR) and gel electrophoresis**

175 Extracted DNA was subjected to PCR for the initial confirmation of *Salmonella* isolates using
176 specific primers (**Table 3**) targeting *invA* gene with the expected amplicon size of 100 bp. A
177 PCR assay was further performed with confirmed 10 *Salmonella* isolates to detect the presence
178 of antimicrobial resistance gene *mcr-1*. In this case, 2 targeting primer sets (**Table 4**) were
179 designed for covering frame reading of antimicrobial (colistin) resistance gene *mcr-1* with the
180 allocated 2 amplicon sizes (1197 bp and 799 bp). Both PCR assays were performed in 20 mL
181 reaction mixture containing 5 uM of each forward and reverse primer and 5 μL of extracted
182 genomic DNA as template. *Salmonella* positive plasmid (5 μL) used as positive controls (PC)
183 and sterile molecular grade water was used as negative controls (NC) to detect cross-
184 contamination during DNA purification and PCR. To optimize PCR reaction, lambda DNA
185 amplification (1000 bp) was used as internal control (IC). PCR was performed using PCR
186 thermocycler (Bio-Rad, United States); conditions for amplification of *invA* and *mcr*-1genes
187 were listed in **Supplementary Table 1 & 2**. The amplified products were visualized by gel

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188 electrophoresis using 1.5% agarose gel and viewed under UV transilluminator in Gel
189 Documentation System (Bio-Rad, United States). For *mcr-1* gene, PCR products of 2 primer sets
190 were purified (Addbio Inc., product code: 10078, South Korea) and used as direct sequencing.
191 Two sequences of each sample was assembled for covering frame reading of *mcr-1* gene and
192 checked with BLAST and annotated to GenBank. In the case of *invA* gene, 10 representative
193 amplicons of *invA* gene fragments were sequenced to confirm the identity of *invA* gene using
194 BLAST (**Supplementary File 1**). DNA sequencing was done by commercial sequencing
195 company SolGent (Daejeon, Republic of Korea).
196
197
198

199 **Sequence acquisition, multiple sequence alignment and phylogenetic analysis**

200 *BLASTp* search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was employed to retrieve the
201 homologous sequences of the MCR-1 and MCR-1 like proteins from the NCBI database using
202 six SAUVM MCR-1 proteins translated from the *mcr-1* genes of *Salmonella spp.* Sequences
203 were carefully categorized into MCR-1 and MCR-1 Like proteins of *Salmonella*, *E. coIi* strains,
204 strains containing LptA (formerly named EptA) and others (**Supplementary File 2 and 3**).
205 Multiple sequence alignment of SAUVM*mcr-1* proteins and retrieved *mcr-1* of *Salmonella*
206 species were performed using T-Coffee with default parameters (34). Maximum Likelihood
207 Method of MEGA X (35) was employed to construct a phylogenetic tree using aligned sequences
208 of MCR-1 from ClustalW (36). Results were validated using 500 bootstrap replicates.

209

210 **Transmembrane topology analysis, structural modelling, refinement and validation**

211 To predict the transmembrane helices of MCR-1 proteins, TMHMM server
212 (http://www.cbs.dtu.dk/services/TMHMM/) was used with standard parameters. The topology
213 was given as the position of the transmembrane helices differentiated by 'i' and 'o' when the
214 loop is on the inside and outside respectively (37). Three dimensional (3D) modelling of
215 SAUVM MCR-1 proteins were performed by I-TASSER which functions by identifying
216 structure templates from the Protein Data Bank (PDB) library. I-TASSER simulations generate
217 large ensemble of structural conformations based on the pair-wise structure similarity. The

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218 confidence of each model is quantitatively measured by C-score (38). To enhance the accuracy
219 of the predicted structures, refinement was performed using ModRefiner (39) followed by FG-
220 MD refinement server (40, 41). Finally, the refined structures were also validated using Verified
221 3D (42, 43), ERRAT (44) and Ramachandran Plot Assessment server (RAMPAGE) (45, 46).

222

223 Molecular docking of PE substrate with *mcr-1* and LptA

224 The chemical structure of Phosphatidylethanolamine (PE) (ZINC identification number [ID]:
225 ZINC32837869) was sampled from the ZINC database (47) while the 3D structure of LptA
226 (PDB ID: 5FGN; Organism: Neisseria meningitidis), the best template of SAUVM MCR-1 were
227 retrieved from the RCSB Protein Data Bank (PDB) server (48). Binding interactions of the PE in
228 the MCR-1 LptA was investigated by molecular docking using Autodock Vina algorithm in
229 PyRx software (49). OpenBabel (version 2.3.1) was used to convert the output PDBQT files in
230 PDB format. PyMol and Discovery Studio software were used to optimize and visualize the
231 protein structures and ligand binding interaction patterns (50, 51).

232

233 Nucleotide Sequence Accession Number

234

235 The sequences of five *mcr-1* genes of *Salmonella* spp. isolated from poultry were deposited into
236 the GenBank database with the Accession No. MN873694, MN873695, MN873696, MN873697
237 and MN873698 for SAUVM_S6, SAUVM_S7, SAUVM_S8, SAUVM_S9 and SAUVM_10,
238 respectively.

239

240 Results

241 Confirmation of *Salmonella* spp. and their antimicrobial susceptibility

242

243 *Salmonella* isolates were confirmed by morphological (Gram's stain), cultural (MacConkey,
244 XLD and S-S agar media) and biochemical (catalase, indole and TSI agar slants test)
245 characteristics as previously described (29). A total of 82 *Salmonella* isolates were differentiated
246 and confirmed following their morphological and biochemical properties. Out of 82 isolates, 10
247 were further confirmed for the presence of *invA* virulence genes, which is accountable for sal-
248 monellosis. PCR (using specific primer listed in **Table 3)** was conducted as a confirmatory

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249 detection tool and found 10 out of 10 (100%) suspected *Salmonella* isolates were *invA* gene (100
250 bp) positive. Amplified DNA fragment of 100 base pairs were considered as positive for
251 *Salmonella* isolates (**Figure 2**). All the *Salmonella* isolates (n=82) were subjected to antibiotic
252 resistance profiling to 15 antimicrobials (**Table 1**). In general, a considerable percentage of
253 resistance was observed across the entire isolates in disk diffusion assay according to the
254 standard of CLSI (31). Specifically, high resistances were found against colistin (92.68%),
255 oxytetracycline (86.59%), co-trimoxazole (76.83%), ciprofloxacin (73.17%) and enrofloxacin
256 (65.85%).Isolates were shown 100% susceptible to the Ceftriaxone (CTR, 30ug), Fosfomycin
257 (FOS,50μg), Norfloxacin (NOR,10μg), Levofloxacin (LEV,5μg), Azithromycin (AZM,15μg),
258 and Neomycin (NEO,10μg) antimicrobials. Only 1 isolate (1/82, 1.22%) was resistant to
259 Lincomycin (LIN, 10μg). Other isolate intermediately resistant to Amoxycilin (AML, 10μg),
260 Streptomycin (STR, 10μg) and Lincomycin (LIN, 10μg). Although, there were some variations,
261 some antibiotic profiles were common among isolates. The multidrug resistant (MDR) patterns
262 were also evaluated among *mcr-1* positive *Salmonella* spp against different antimicrobial classes.
263 All the isolates (100%) showed MDR against 3 antimicrobial classes (colistin, oxytetracycline
264 and ciprofloxacin) (**Table 2**).
265
266
267 **Detection of colistin resistance *mcr-1* gene**
268 *Salmonella* isolates were further evaluated for the presence of the *mcr-1* gene through PCR using
269 *mcr-1* specific primers (**Table 4**). Five samples were likely to carry colistin resistance *mcr-1*
270 among 10 randomly selected *Salmonella* isolates obtained from chickens (**Figure 3**). In Sanger
271 sequencing demonstration, sequence found 100% identical with *mcr-1* gene accessed in the
272 NCBI database and *mcr-1* gene described by Liu et al. (2016).
273
274 **Sequence acquisition, multiple sequence alignment and phylogenetic analysis**
275 In order to analyze sequence similarities, phylogeny and structural insights of *mcr-1* gene
276 products, the respective translated MCR-1 proteins were employed in different bioinformatics
277 studies. A total of 52 homologous sequences of the MCR-1 and MCR-1 like proteins were
278 retrieved from the NCBI database, while 44 sequences were employed to phylogenetic analysis
279 including SAUVM-MCR-1 proteins. Again, 8 MCR-1 proteins of *Salmonella* spp were aligned

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280 with SAUVM-MCR-1 proteins for further analysis. The evolutionary relation inferred via
281 phylogeny analysis has been given in **Figure 4**. The phylogenetic analysis revealed that, all of
282 the MCR-1 and MCR-1 like proteins were distinctly categorized into 2 major groups;
283 chromosomally-encoded LptA and plasmid encoded MCR types, indicating a divergent
284 evolutionary relation between the LptA and MCR proteins.

285

286 **Transmembrane topology analysis, structural modelling, refinement and validation**

287 Prediction of transmembrane helices is of utmost importance in functional analysis of protein.
288 Therefore, TMHMM server was applied for predicting transmembrane helices in mcr-1 genes of
289 *Salmonella* isolates. TMHMM predicted that, there were five transmemebrane domain in the
290 SAUVM-MCR-1 proteins; TMhelix1 (13-35), TMhelix 2 (50-72), TMhelix 3 (79-101), TMhelix
291 4 (123-145) and TMhelix 5 (158-180) which were spanned in the inner membrane region
292 (**Figure 5**). The structure of SAUVM-MCR-1 proteins were modeled using I-TASSER server,
293 where *N. meningitis* EptA (PDB ID 5FGN) acted as the structural template. SAUVM-MCR-1
294 proteins showed 35.4% (35.6%) identity to EptA, and their modelled structure possesses a
295 coverage score of 96% compared with that of EptA. Refinement was performed to enrich the
296 quality of predicted structures beyond the accuracy. After refinement Ramachandran plot
297 analysis revealed that, 83.3% residues were in the favored, 12.4% residues in the allowed while
298 only 4.3% residues were in the outlier region (**Figure 6**). Moreover, ERRAT showed 94.4%
299 quality factor (**Supplementary Figure 1A and 1B**) and Verify3D suggested that, 94.74% of the
300 residues had averaged 3D-1D score >= 0.2 (**Supplementary Figure 2**).

301

302 **Molecular docking of PE substrate with MCR-1 and LptA**

303 The grid box was set to 82.0138A° x 82.7041A° x 82.471A° (x, y and z) with 1 A° spacing
304 between the grid points, while other parameters were default. Though, molecular docking of PE
305 substrate with SAUVM-MCR-1 and LptA generated five docking binding conformation for each
306 but the binding pattern with lower energy had been selected (PE & MCR-1: -3.4kcal/mol and PE
307 & LptA: -3.6 kcal/mol). Again, it was demonstrated that Leu 64, Tyr 179 and Phe 183 were the

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308 key interactive molecules in PE binding cavity of SAUVM MCR-1 whereas Ser 61, Tyr 174, Phe
309 181, Val 192 and Ser 194 were for LptA (**Figure 7**).

310

311

312 **Discussion**

313

314 *Salmonella* is the primary and leading cause of food borne diseases globally; accounting for 78
315 million affected and 59 thousand deaths annually (52, 53). This is an endemic food borne disease
316 in South Asian countries like Bangladesh (54, 55). A broad range of food stuff has been
317 associated with such illness. Among them, food from animal sources, especially poultry is in
318 main list. Due to its potential to cause enteric disease, the detection of *Salmonella* isolates in
319 poultry is of great concern which are globally recognized as food borne zoonoses (52, 55, 56).
320 The severity and length of such diseases could reduce by antimicrobial therapeutics in human
321 (53) and poultry as well (57, 58). However, the rising of antimicrobial-resistant that is,
322 antimicrobials commonly prescribed to treat *Salmonella* are losing its ability to stop growing or
323 killing of *Salmonella* has become a significant public health issue now days (Islam and Shiraj-
324 um-mahmuda, 2016; Ahmed et al., 2019 Wasyl et al., 2015; Iwamoto et al., 2017). As a result,
325 standard treatment become ineffective, infections persist, and may increases chance of spreading
326 to others (53, 61). Therefore, antimicrobial resistance is of great concern and challenging for any
327 country. So far, Bangladesh requires baseline data on resistant bacteria like *Salmonella* and their
328 molecular detection from various sources (e.g. poultry) to develop effective strategies against
329 antimicrobial resistance and its hazards.

330

331 In the present study, to explore morphological, biochemical and molecular detection of
332 antimicrobial resistant *Salmonella* and their genes in poultry, samples were obtained from both
333 broiler and layer birds that were dead or clinically sick. Initial screening for infection has been
334 done on the basis of clinical history, signs and postmortem findings. Among 100 suspected
335 samples from different poultry zones of the country, 82 found positive for *Salmonella* spp. which
336 was not unexpected as previous studies found that, even apparently healthy commercial poultry
337 and their surrounding environment can carry *Salmonella* spp. in Bangladesh (62, 63).

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338 Molecular methods were optimized for rapid identification of *Salmonella* isolates and its
339 resistance genes in poultry using specific primers followed by nucleotide sequencing and
340 phylogenetic analysis. The virulence of *Salmonella* is linked to a combination of various factors,
341 for example, *invA* virulence gene in the inner membrane of bacteria that are necessary for
342 invasion of epithelial cells (18, 19). In this study, detection of virulence *invA* gene (10 out of 10
343 isolates) in the isolated *Salmonella* indicates the pathogenic nature of these isolates.
344 Resistance of *Salmonella* spp. to antimicrobials is an emerging threat in developing as well as
345 developed countries (7). It is therefore necessary to determine the resistance patterns of isolates
346 to minimize resistance hazards. In this study, antibiotic susceptibility results showed 100% of the
347 *Salmonella* isolates were resistant to colistin and oxytetracycline. This high resistance rates
348 reflect widespread use of these antibiotics in animal feed and are consistent with other reports
349 (25, 64). And rate of resistance to ciprofloxacin (80.49%) and enrofloxacinin (74.39%) deserves
350 attention because *Salmonella* spp. resistance to these antibiotics may cause human infection too
351 (58). However, previous studies reported that, *Salmonella* isolates were sensitive to ciprofloxacin
352 (7, 62, 65). Similar results were observed in amoxicillin and doxycycline antimicrobials. These
353 antimicrobials resistance against *Salmonella* isolates might designate the over use or abuse of
354 these antibiotics (14, 15, 58). On the other hand, *Salmonella* isolates found to be susceptible to
355 ceftriaxone, fosfomycin, neomycin, levofloxacin, norfloxacin and azithromycin which is in line
356 with previous reports (64, 66). It may be because; these antibiotics are not commonly used for
357 therapeutic purposes in veterinary medicine. . The present study represented that, 100% of the
358 *mcr-1* positive isolates were MDR. Similar findings were reported on MDR in *Salmonella*
359 isolates from Bangladesh and different parts of the world (29, 67–72). Due to the indiscriminate
360 victimization of antimicrobial agents, MDR strains may apparently be occurred with high
361 incidence in this area which is a serious concern for veterinary medicine and also for human
362 health since direct transmission of resistant isolates from animals to humans has been confirmed
363 (73).
364
365 Since early 1980s, colistin has been widely used in agricultural sector in China (25, 74), caused
366 the initial emergence and spread of *mcr-1* worldwide (25, 68, 75, 76). In this study, an
367 unexpected presence of (5 out 10 samples) colistin resistance *mcr-1* gene was detected in
368 *Salmonella* spp. from poultry specimens (e.g. liver, intestine). This higher rate of *mcr-1* in

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369 chicken *Salmonella* isolates was surprising and suggested that *mcr-1* might already be
370 widespread in food animals in Bangladesh. However, we do not have actual data on
371 antimicrobial usage on the farms where the samples originated. Therefore, the presence of *mcr-1*
372 gene may suggest frequent use of colistin and possibly other antimicrobials in the poultry
373 industry in this region. A recent study has been reported 28% of poultry samples harbored *mcr-1*
374 in China which has linked between human and animals (25). Subsequent study found an
375 unexpected high prevalence (24.8%) of *mcr-1* in retail chicken meat samples in Netherlands
376 (77). Following that work, investigations by other scientists have confirmed the presence of *mcr-*
377 *1* in *Salmonella* isolates recovered from mussels and poultry (77–80). The high prevalence of the
378 *mcr-1* gene in *Enterobacteriace* isolates of poultry is certainly concerning for a country like
379 Bangladesh where antimicrobial use in both human and animals may be poorly regulated.
380 Therefore, the recent emergence of colistin resistance has triggered an international review and
381 recommendations for restrictions of colistin use in farm animals (68, 81).
382
383 While, detailed study on molecular mechanisms of antimicrobial resistance is lacking, we aimed
384 to address *mcr-1* using integrative approaches ranging from nucleotide analysis, bioinformatics
385 and structural modeling of bacterial genetics. The detection of new *mcr-1*-harbouring *Salmonella*
386 isolates adds new knowledge to the newly-emerging issue of colistin resistance *mcr-1* genes. It
387 furthering our understanding on homology, structure and validation of the *mcr-1* genes present in
388 *Salmonella* isolates. It was reported that, colistin resistance *mcr-1* gene is present in a multidrug-
389 resistant plasmid (82) and *mcr-1* genes of this study found somewhat similar to a recently-
390 isolated plasmid from China (25). These facts imply that, multidrug resistant bacteria with
391 colistin resistance will eventually evolve a fact that deserves close attention.
392
393 Thus, we are awfully interested in determining the multiple sequence alignments for *mcr-1* genes
394 in *Salmonella* spp. isolated from poultry. In order to avoid hits from very closely related species,
395 retrieved sequences of *Salmonella* species were excluded from the phylogeny study and those
396 were only aligned with SAUVM *mcr-1* proteins. The multiple sequence alignments of SAUVM
397 *mcr-1* proteins clearly indicated that, they belongs to the Mobilized Colistin Resistance (MCR)
398 protein family with putative conserved sites [**Supplementary File 2**].
399

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400 We experimentally validated the expression of colistin resistance *mcr-1* of *Salmonella* isolates
401 (**Figure 3A and 3B**), suggesting the possibility of evolutionary path for the *mcr-1* genes.
402 To address this concern, we conducted phylogenetic analyses. The phylogeny indicates a
403 divergent evolutionary pattern between the LptA and MCR-1 including MCR-1 like proteins.
404 The constructed phylogenetic tree provides information about ancestral origin and diversification
405 of the MCR-1 proteins in different organism divided into chromosomally-encoded LptA and
406 plasmid encoded MCR types indicating a divergent evolutionary relation between the LptA and
407 MCR proteins (Figure 4). Further, MCR proteins group were divided into 2 apparent subgroups,
408 one of which features MCR-1 proteins mostly of *E. coli* strains including SAUVM MCR-1 and
409 the other one compromising small subclade of MCR-2 with MCR-1 proteins from diverse
410 organisms. All of the SAUVM MCR-1 proteins were closely related to the *E. coli* MCR-1
411 sharing the position in the same clade. However, SAUVM MCR-1 and LptA fall into 2 separate
412 subclades within the tree which indicated the low sequence identity, also reported by previous
413 studies (83, 84). Despite the fact that, *E. coli* MCR-2 proteins were mostly aligned with MCR-1
414 proteins of non *E. coli* groups. Again, the Z1140 locus of *E. coli* O157:H7, a member of the
415 PEA lipid a transferases lacking a role in colistin resistance apparently formed individual clade
416 in the phylogeny which strengthened the findings. For understanding the structural insight, 3D
417 homology modeling of five of SAUVM MCR-1 proteins were constructed using Neisseria
418 meningitides LptA as structural template and five distinct transmembrane helices spanned in the
419 inner membrane region were identified which was also reported by different studies (68, 84).
420 Again, molecular interactions between Phosphatidylethanolamine (PE) substrate with MCR-1
421 and LptA had been investigated as colistin resistance proteins. MCR-1, MCR-2 and LptA were
422 found to share similar PE lipid substrate-recognizing cavity. Ligand-binding interaction pattern
423 of PE substrate with SAUVM-MCR-1 and LptA revealed that, both proteins exhibited similar
424 localization of PE binding sites spanning from 175-195 region in which Phe, Tyr and Ser
425 residues were abundantly found.
426
427 The data we present represents a first comprehensive glimpse of antimicrobial (colistin)
428 resistance *mcr-1* genes among poultry originated *Salmonella* isolates in Bangladesh. This study
429 provides further substantial evidence for the need of implementation of risk-management

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430 strategies and the need to review the extensive use of colistin in food animals for urgently
431 advocated and implemented in this country.
432
433
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711 Figure legends

712 **Figure 1: A map of Bangladesh showing locations of the sampling sites under selected**
713 **districts.** The areas where the *mcr-1* gene has been screened are highlighted in red.

714 **Figure 2: PCR amplification of *invA* gene of *Salmonella* isolates.** In all isolates (S1~S10), a
715 fragment of 100 bp (*invA* gene) was detected. Lane M: DNA ladder. Lane PC: positive control
716 for *invA* gene; Lane NC: negative control for *invA* gene; Lanes S1-S10, amplified gene of *invA* in
717 the tested isolates. [S1~10=*Salmonella* isolate 1~10].

718 **Figure 3: PCR amplification of antimicrobial (colistin) resistance *mcr-1* gene of *Salmonella***
719 **isolates.** In *Salmonella* (S6~S10) isolates a fragment of (A) 1197 bp and (B) 799 bp was
720 detected. Lane M: DNA ladder. Lane NC: negative control for *mcr-1* gene. [S6~10=*Salmonella*
721 isolate 6~10].

722 **Figure 4: Phylogeny analysis showing ancestral origin and diversification of MCR-1 and**
723 **MCR-1 like proteins**. *BLASTp* search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was employed to
724 retrieve the homologous sequences of the MCR-1 and MCR-1 like proteins from the NCBI
725 database using amino acid sequences of six SAUVM-MCR-1 proteins. Sequences were
726 carefully categorized into the MCR-1 and MCR-1 Like proteins of *Salmonella, E. coli* strains,
727 strains containing LptA (formerly named EptA) and others. Maximum Likelihood Method of
728 MEGA X was employed to construct a phylogenetic tree using aligned sequences of MCR-1
729 from CLUSTALW.

730 **Figure 5: Transmembrane topology prediction of SAUVM-S6-MCR-1 protein.** TMHMM
731 server (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict the (**A**) transmembrane

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732 helices of MCR-1 proteins. (**B**) The topology was given as the position of the transmembrane
733 helices differentiated by 'i' and 'o' when the loop is on the inside and outside, respectively.

734 **Figure 6: Modelled structures of SAUVM-MCR-1 proteins and Validation.** (**A**) Three
735 dimensional (3D) modelling of SAUVM-MCR-1 proteins (SAUVM_S6~S10) were performed
736 by I-TASSER which functions by identifying structure templates from the PDB library. The
737 confidence of each model is quantitatively measured by C-score. From these models of MCR-1
738 proteins, SAUVM_S10 model was randomly selected, (**B**) analysed and structures validated with
739 Ramachandran Plot Assessment server (RAMPAGE).

740 **Figure 7: Ligand-binding interaction pattern of PE substrate with colistin resistance MCR-**
741 **1 and LptA.** The modeled ribbon structure for PE substrate with MCR-1 protein. The ribbon
742 structure was given via PyMol software. In both (**A**) and (**B**) cases, the substrates tend to bind in
743 the groove of MCR-1 and LptA mostly spanning from 175-195 region, in which Phe, Tyr and
744 Ser residues were abundantly found in the substrate binding region for PE interaction. (**B**)
745 Ligand-binding interaction revealed that both MCR-1 and LptA proteins exhibited similar
746 localization of PE binding sites (SAUVM-MCR-1: Leu 64, Tyr 179, Phe 183; LptA: Ser 61, Tyr
747 174, Phe 181, val 192, Ser 194).

748

749 **Supplementary Files**

750 **Supplementary File 1:** Amplicons of 100bp *invA* gene sequence of *Salmonella* isolates
751 (S6~S10).

752 **Supplementary File 2:** Retrieved sequences of MCR-1 and MCR-1 like proteins.

753 **Supplementary File 3:** The multiple sequence alignments of SAUVM-MCR-1 proteins with
754 putative conserved sites of other *Salmonella* MCR-1.

755 **Supplementary File 4:** Supplementary Table S1, S2 and S3.

756 **Supplementary File 5:** Structure validation by ERRAT and Verify3D server.

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Figure 1

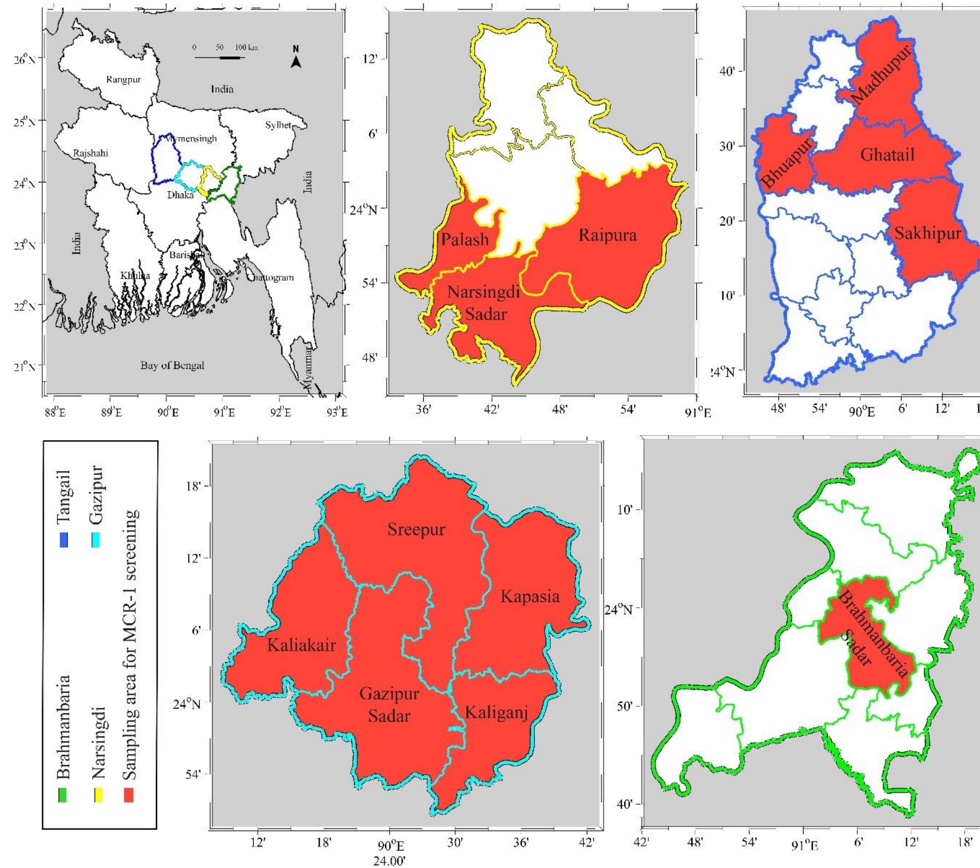


Figure 1: A map of Bangladesh showing locations of the sampling sites under selected districts. The areas where the *mcr-1* gene has been screened are highlighted in red.

Figure 2

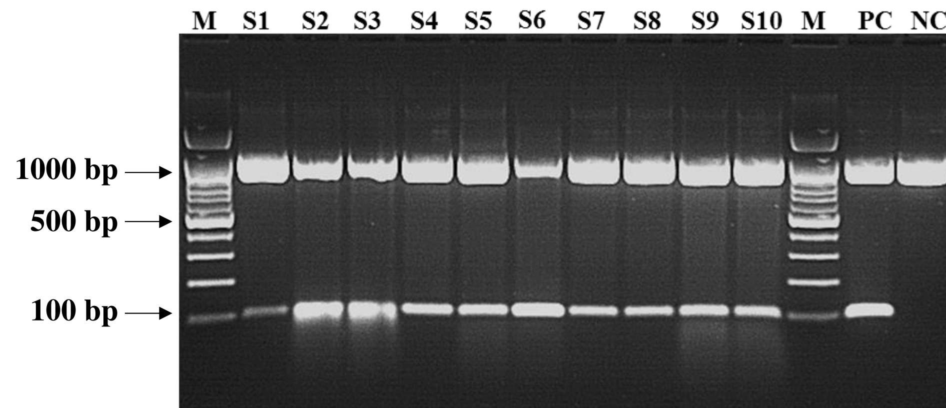


Figure 2: PCR amplification of *invA* gene of *Salmonella* isolates. In all isolates (S1~S10), a fragment of 100 bp was detected. Lane M: DNA ladder. Lane PC: positive control for *invA* gene; Lane NC: negative control for *invA* gene; Lanes S1-S10, amplified *invA* gene in tested isolates. [S1~10=*Salmonella* isolate 1~10]

Figure 3

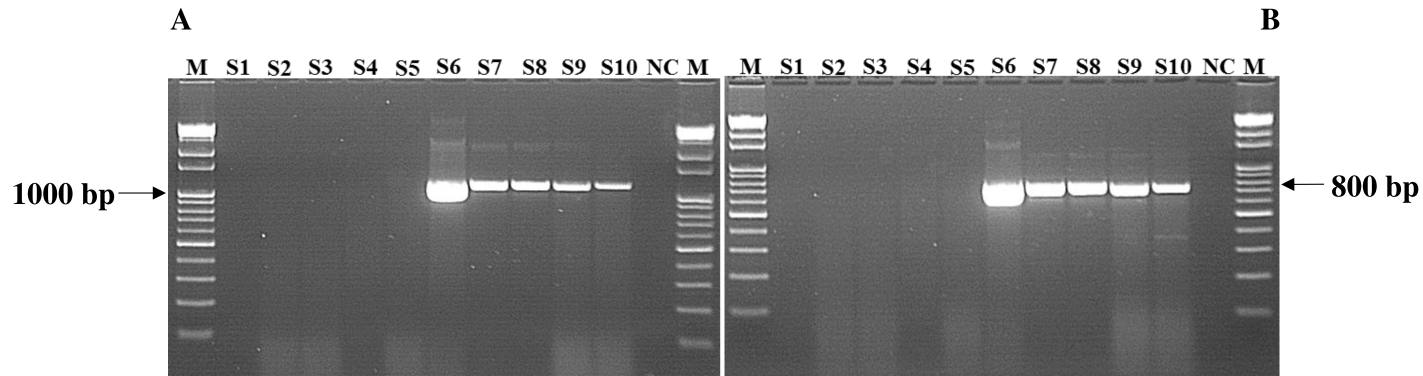


Figure 3: PCR amplification of antimicrobial (colistin) resistance *mcr-1* gene of *Salmonella* isolates. Amplified gene of *mcr-1* in the tested isolates. In *Salmonella* (S6~S10) isolates, (A) a fragment of 1197 bp; and (B) a fragment of 799 bp was detected. Lane M: DNA ladder. Lane NC: negative control for *mcr-1* gene. [S6~10=*Salmonella* isolate 6~10]

Figure 4

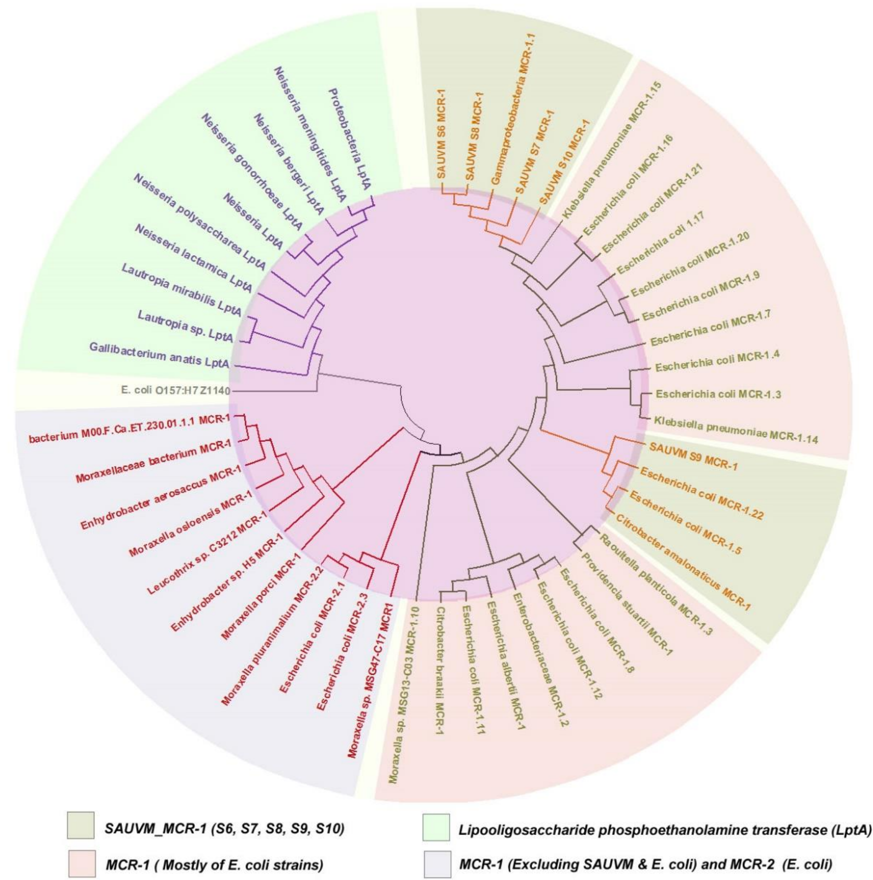
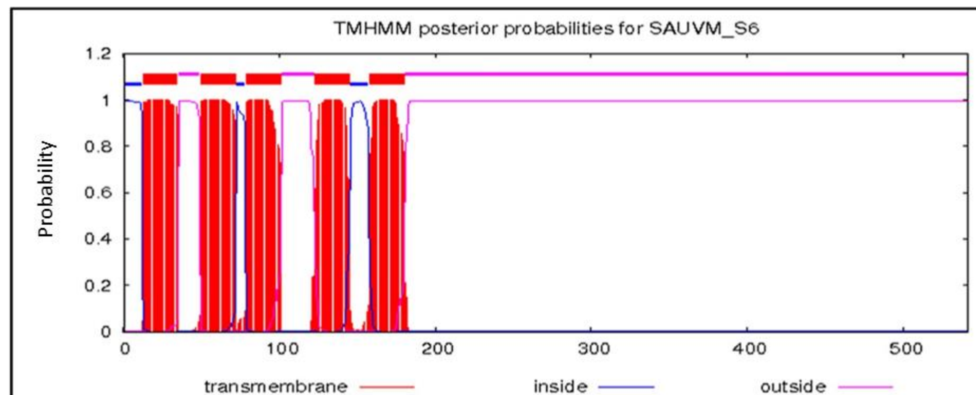
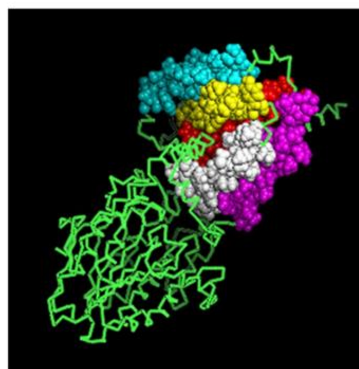


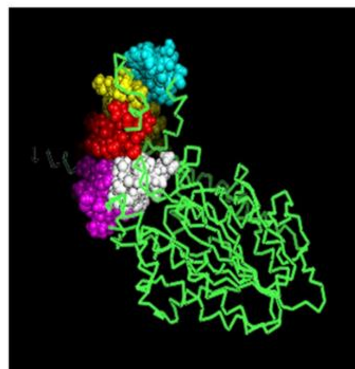
Figure 4: Phylogeny analysis showing ancestral origin and diversification of MCR-1 and MCR-1 like proteins

Figure 5



(A)





TMhelix 1(13-35)

TMhelix 2(50-72)

TMhelix 3(79-101)

TMhelix 4(123-145)

TMhelix 5(158-180)

(B)

Figure 5: Transmembrane topology prediction of SAUVM_S6-MCR-1 protein

Figure 6

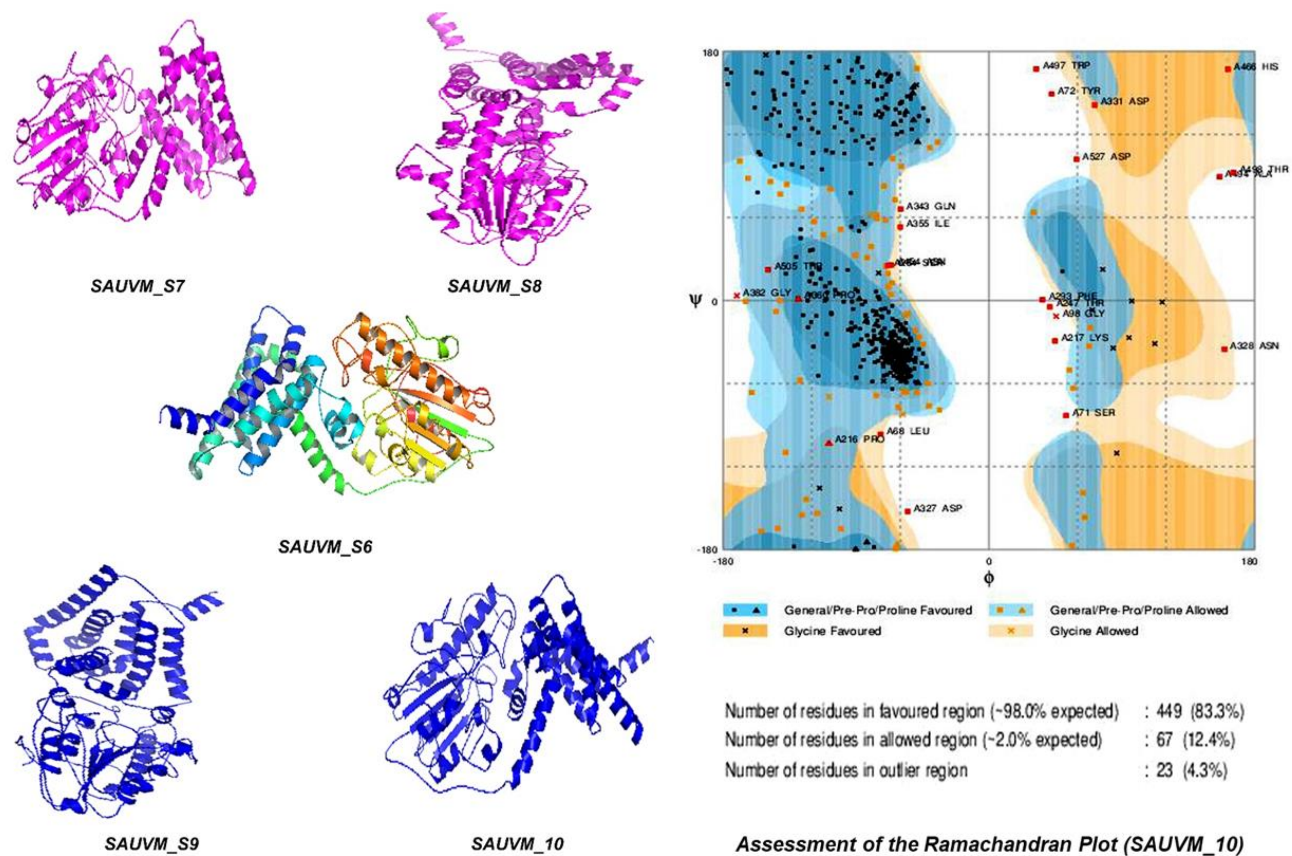


Figure 6: Modelled structures of SAUVM-MCR-1 proteins and validation

Figure 7

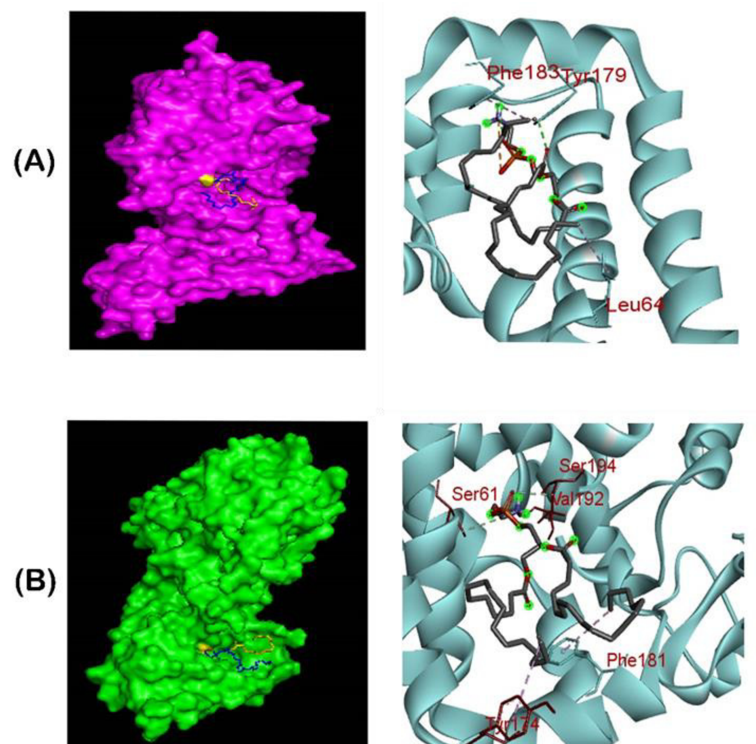


Figure 7: Ligand-binding interaction pattern of PE substrate with MCR-1 and LptA

Table 1: Antimicrobial susceptibility pattern of *Salmonella* isolates (n=82).

Antibiotics	Susceptible (S)		Intermediate (I)		Resistant (R)		I+R
	No. of isolates	%	No. of isolates	%	No. of isolates	%	
Penicillins /β-Lactamase inhibitor							
Amoxycilin (AML,10µg)	29	35.37	48	58.54	5	6.10	64.63
Mcrolides							
Azithromycin (AZM,15µg)	80	97.56	2	2.44	0	0.00	2.44
Aminoglycosides							
Streptomycin (STR,10µg)	25	30.49	45	54.88	12	14.63	69.51
Neomycin (NEO,10µg)	82	100.00	0	0.00	0	0.00	0.00
Quinolones							
Ciprofloxacin (CIP,5µg)	16	19.51	6	7.32	60	73.17	80.49
Levofloxacin (LEV,5µg)	82	100.00	0	0.00	0	0.00	0.00
Fluoroquinolones							
Enrofloxacin (ENR,10µg)	21	25.61	7	8.54	54	65.85	74.39
Norfloxacin (NOR,10µg)	82	100.00	0	0.00	0	0.00	0.00
Tetracyclins							
Oxytetracycline (OTC,30µg)	3	3.66	8	9.76	71	86.59	96.34
Doxycycline (DOX,30µg)	27	32.93	16	19.51	39	47.56	67.07
Lincosamide							
Lincomycin (LIN,10µg)	59	71.95	22	26.83	1	1.22	28.05
Phosphonic							
Fosfomycin (FOS,50µg)	82	100.00	0	0.00	0	0.00	0.00
Folate Pathway Inhibitor							
Co-trimoxazole (COT,25µg)	9	10.98	10	12.20	63	76.83	89.02
Polymyxins							
Colistin (COL,10µg)	0	0.00	6	7.32	76	92.68	100.00
Cephalosporin							
Ceftriaxone (CTR,30ug)	82	100.00	0	0.00	0	0.00	0.00

% = percentage (number of isolates/total number of isolates tested).

Table 2: Multidrug resistant patterns among the *mcr-1* positive *Salmonella* spp isolated from poultry farms in Bangladesh

Salmonella isolates	Multidrug resistant patterns	No. of antibiotic classes to which the tested isolates exhibits resistance (n = 7)
S-6	COL+OTC+COT+CIP+STR	5
S-7	COL+OTC+COT+CIP	4
S-8	COL+OTC+AZM+CIP+STR	5
S-9	COL+OTC+COT+CIP	4
S-10	COL+OTC+COT	3

COL = Colistin, OXT = Oxytetracycline, AZM= Azithromycin, COT= Co-trimoxazole, CTR=Ceftriaxone, CIP = Ciprofloxacin, STR = Streptomycin.
Resistant if, Colistin: ≤ 10; Tetracycline: ≤ 11; Azithromycin:≤ 12; Ceftriaxone: ≤ 14; Ciprofloxacin: ≤ 20; Streptomycin: ≤ 11.
Multidrug resistant patterns was determined according to the recommendations by CLSI zone diameter interpretation criteria (nearest whole mm).

Table 3: Primer sequence for *invA* gene detection in *Salmonella* isolates

Target gene	Primer name	Primer sequence (5' – 3')	Amplicon Size (bp)
Lambda (Internal control)	LF	CAGATCTCCAGCACGGAACTATTGAGTACGAACG	1000
	LR	GCATAAAATGCGGGGATTCACTGGCTGC	
invA	SF	TAATGCCAGACGAAAGAGCGT	100
	SR	GATATTGGTGTTTATGGGGTCGTT	

Lambda primer: In 20 ul PCR reaction forward and reverse primer used as 10 uM each and lambda DNA 500 pg

Table 4: Primer sequence for *mcr-1* gene detection in *Salmonella* isolates

Target gene	Primer name	Primer sequence (5' – 3')	Amplicon Size (bp)
mcr-1	mcr1-P1F	CAGTATGGGATTGCGCAATGATT	1197
	mcr1-P1R	TTATCCATCACGCCTTTTGAGTC	
mcr-1	mcr1-P2F	TGTCGATACCGCCAAATACCAAG	799
	mcr1-P2R	GGAGTGTGCGGTGGGTTTG	