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Molecular detection of *mcr-1* gene of *Escherichia coli* isolated from infected broiler chickens

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ABSTRACT

Avian colibacillosis is one of the most devastating diseases in chickens, resulting in high economic losses as well as high morbidity and mortality. Colistin is considered one of the most effective antimicrobials against colibacillosis. The recent discovery of mobile colistin resistance (*mcr-1*) determinants in humans and animals raised serious concerns about its potential dissemination among bacteria. This study aimed to investigate the presence of *mcr-1* gene among *E. coli* isolates in broiler chickens infected with colibacillosis. As an experimental design was performed on broiler chickens after molecular detection of *mcr-1* gene to explore the efficacy of neomycin as a hydrophobic antibiotic wither in the *mcr-1* absence or its existence after oral inoculation of *E. coli* strains. Results revealed the high prevalence of colibacillosis among broiler farms in addition to highly spread of colistin resistance among *E. coli* isolates either through isolation on specific colistin resistance medium or molecular detection of the *mcr-1* gene. On other hand, by histological examination we observed that the presence of the *mcr-1* gene among *E. coli* isolates improved the treatment efficacy of the hydrophobic antibiotic. As a result of the significant antibiotic resistance issues and high risk of transmission of antibiotic-resistant bacteria and genes to humans, the development of antibacterials only for animal uses without cross-resistance to existing antibiotics might afford a solution in the future. Although there is an increase in acquired antibiotic resistance genes, it is possible to take advantage of this phenomenon, which in turn led to an increase in the sensitivity of some other antibiotics. Therefore, we recommend studying pharmacokinetic of antibacterials and making the most of this widespread phenomenon.

Introduction

Avian Pathogenic *Escherichia coli* (APEC), is a Gram-negative, rod-shaped extra-intestinal pathogen, causes a clinical disease called colibacillosis, which is considered one of the most commonly occurring and economically devastating diseases of poultry worldwide that reaches hundred millions of dollars, because of carcass condemnation which is up to 43%, morbidity and mortality range from 20 to 53.5% in young chickens (Ahmed *et al*., 2013; Mellata, 2013; Ghunaim *et al*., 2014)

Colibacillosis represents two-thirds of the reported bacterial infections in different types of poultry production (Souillard *et al*., 2011), resulting in local and systemic infections such as septicemia, omphalitis, swollen head syndrome, cellulitis, pericarditis and perihepatitis (Paixao *et al*., 2016). Several APEC serotypes have been detected with colibacillosis in the outbreaks; but, O78, O2, and O1 represent up to 80% of the most common field cases (Ghunaim *et al*., 2014). Antibiotics have been used as the main tool in contradiction of colibacillosis for many decades. However, the emergence of increased antibiotic resistance has dictated the need for improved treatment as well as robust preventive measures to avoid the disease.

Although polymyxin have regained popularity as a last alternative treatment to confront the worldwide emergence of Gram negative bacteria (Otsuka, 2020), the abuse of colistin is facing a globally increasing resistance, that denotes a clinical source of concern, due to the plasmid-encoded mobilized colistin resistance (*mcr-1*) gene presented in Enterobacteriaceae such as *Escherichia coli* and Klebsiella pneumoniae ,described in 2015, and detected in humans and livestock globally (Nang *et al*., 2019) results in a moderate level of resistance, with MIC values 4 to 16 mg/l ml (Skov and Monnet, 2016; Zeng *et al*., 2016)

Mcr-1 is a membrane-bound enzyme containing a soluble form in the peri-plasmic space and five hydrophobic trans-membrane helixes (Liu *et al*., 2016). Colistin resistance of *mcr-1* gene is conferred by encoding a phosphoethanolamine transferase enzyme which catalyzes the addition of a phosphoethanolamine moiety to lipid A in the bacterial outer membrane (OM) (Gao *et al*., 2016; Hinchliffe *et al*., 2017), that may alter the structure of lipid A that led to decrease in growth rate and the cell viability, competitive ability and the shape of cytoplasmic structures (Yang *et al*., 2017).

Bacterial cell protection against an adverse environment and exchanging material critically is done by the OM of Gram-negative pathogens (Costerton *et al*., 1974); thus, to be efficient, the inhibitory concentration of antibiotics must pass across the OM barrier to reach inside the bacterial cell (Vergalli *et al*., 2017). So, the low permeability of bacterial OMs is considered a strong barrier that inhibits many antibiotics reaching intracellular target, as they usually cross the OM either by the lipid-mediated path responsible for hydrophobic antibiotics, such as aminoglycosides (gentamycin, kanamycin and neomycin) and macrolides or by the general distribution porins for hydrophilic antibiotics such as ß-lactams (Nikaido, 2003).

Since *mcr-1* modifies the structure of LPS in the bacterial OM mutants by altering the membrane permeability, this may increase the susceptibility of bacteria to most hydrophobic antibiotics (Gao *et al*., 2016).

Therefore, the current study aimed to molecular investigation of plasmid mediated colistin resistance (*mcr-1*) in *E. coli* isolates and in vivo evaluation of the treatment effect with neomycin as a hydrophobic antibiotic in the presence of the *mcr-1* gene.

Materials and methods

Ethics approval and consent to participate

All the samples were collected under the permission in accordance

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with the local license. All experiments were performed in experimental units of Avian and Rabbit Medicine Department, Faculty of Veterinary Medicine, Assiut University, and approved by Ethical Committee of Veterinary Medicine Faculty, Assiut University, Assiut, Egypt (Protocol number aun/vet/3/0005) according to the standards of OIE for use of animals in research in accordance the relevant guidelines and regulations. Informed consent was obtained from all owners' poultry farms.

Sampling

A total of 200 samples were equally collected from the liver and hearts of freshly dead and diseased broiler chickens that, before being euthanized, were anesthetized by intramuscular injection of both xylazine 5 mg/kg (ADWIA Co.) and ketamine (10%) 35 mg/kg (Bremer Pharma Gmbh) (Bergfeld *et al*., 2017) as they became unconscious and dead (OIE, 2018), from different poultry farms in Assiut governorate, aged from 1 to 35 days old suspected to be infected with colibacillosis.

Isolation and Identification of E. coli (Quinn et al., 2011)

Each sample was inoculated separately into brain heart infusion broth and incubated at 37°C for 24 h in aerobic condition. Then subculture was made on selective differential solid media onto MacConkey's agar and Eosin Methylene blue agar. The cultured plates were incubated at 37ºC overnight. Suspected *E. coli* purified colonies were kept for identification using biochemical reactions, e.g. Indole test, Triple Sugar Iron Agar (TSI), Kosar citrate utilization and Catalase test.

Detection of colistin resistant E. coli

Universal Culture (super polymyxin) Media

Super polymyxin media was prepared according to Nordmann (2016a), (15 mg EMB agar powder dissolved in 400 ml Distilled water), with the addition of 200µl Lincomycin from the prepared stock solution (20 mg in 1ml D. W), 70µl Colistin sulphate (20 mg in 1ml D. W), and 100µl mycostatin (ATECO Company. Diluted EMB powder was autoclaved at 121°C for 20 min. and antibiotics were added after water bathing the medium for 1h at 56°C.

Rapid polymyxin NP test

This test relay on production of acid that identified by phenol red PH indicator from carbohydrate metabolism of Gram negative bacteria. This test was performed according to Shoaib *et al*. (2020), incubated at 37°C and inspected from 1 to 4 h.

Molecular Confirmation of E. coli

Extraction of DNA

DNA from overnight cultured broth was extracted by boiling. Brain heart infusion broths were centrifuged at 15,000 rpm for 15 min. The supernatant was discarded, and then the pellet was re-suspended in grade water and centrifuged at 15,000 rpm for 10 min. The pellets were eluted in 40µ grade water, subjected to boiling at 100°C in a dry water bath for 20 min, freeze on ice, and centrifuged at 15,000 rpm for 10 min. Aliquots of template DNA were stored at -20°C for PCR (Queipo-Ortuño *et al*., 2008).

Primers set

Primers are specific to *pho*A gene (Hu *et al*., 2011) that supposedly gives amplified product at 720bp. Primer forward 5-CGATTCTGGAAATGGCAAAAG -3 and reverse primer 5- CGTGATCAGCGG TGACTATGAC-3. Primers are specific to colistin mediated resistant gene (*mcr-1* gene) (Rebelo *et al*., 2018), with amplified amplicon (320bp) Primer forward 5-AGTCCGTTTGTTCTTGTGGC -3 and reverse primer 5- AGATCCTTGGTCTCGGCTTG-3.

All PCR reactions were carried out in a final volume of 25µl containing 12.5 µl Go Tag® Green master mix (Promega Corporation USA), 1 µl forward primer, 1 µl reverse primer, 4 µl DNA template and 6.5 µl DNase/RNase-free water.

PCR condition

PCR cycling condition was carried out as following: 95°C for 5 min., followed by 35 cycles at 94°C for 45 sec, annealing temperature 58°C (*mcr-1* gene), 55°C (*pho*A gene) for 45 sec and 72°C for 45 sec and then a final extension at 72°C for 10 min then PCR products were visualized by gel electrophoresis. A reference strain of *E. coli* (O78:K80), was used as a positive control and was provided by Avian and Rabbit Medicine Department, Faculty of Veterinary Medicine, Assiut University.

Serological identification of E. coli isolates with and without mcr-1gene

Isolates were serologically identified in Food Control Department; Faculty of Veterinary Medicine in Moshtar, Banha according to Kok *et al*. (1996) by using rapid diagnostic *E. coli* antisera sets (DENKA SEIKEN Co., Japan).

Experimental design

Serologically tested *E. coli* isolates that have been used in vivo belong to O91 (with *mcr-1*) and O78 (without *mcr-1*).

One hundred and fifty one day old Cobb broiler chicks (El-Waddi Co, Egypt) were received at one day old kept in an experimental room of Avian and Rabbit Medicine Department, Faculty of Veterinary Medicine, Assiut University, Egypt. Broilers were divided into 2 groups 60 chicks each group, in addition to the third control group (not infected and not treated) contained 30 chicks. Groups 1 and 2, on the 7thday of age was challenged orally with 0.5 ml of 4×10^9 CFU /ml *E. coli* strains O91 (with *mcr-1* gene), and O78 (without *mcr-1* gene), respectively. After 3 days post infection both groups 1(O91) and 2(O78), were subdivided equally into group 4(O91) and group 5(O78), respectively. Groups 1 and 2 remained without any treatment till the end of the experiment to study the pathogenicity of the *E. coli* strains, in the other hand, both groups 4 and 5 were treated with neomycin), (14% W.S.P. ATCO PHARMA) in drinking water (1g/liter) for 5 days begin from the 10th day till the 14th days of age.

After 3 days post infection both groups 1 and 2 were subdivided into groups 4 and 5 correspondingly, to be treated with neomycin, (14% W.S.P. ATCO PHARMA) in drinking water (1g/liter) for 5 days begin from the 10th day till the 14th days of age.

All groups were kept under observation till the end of the study for signs, postmortem inspection of dead and euthanized diseased chickens, as they became unconscious and dead (OIE, 2018), after being anesthetized by intramuscular injection of both xylazine 5 mg/kg (ADWIA Co.) and ketamine (10%) 35 mg/kg (Bremer Pharma Gmbh) (Bergfeld *et al*., 2017), mortality rate, and histopathological inspection.

Histopathological examination

Specimens of liver and heart from 3 freshly dead and euthanized chickens were collected from each group, on the 3rd and 8th days post infection (dpi) and the 5th day post treatment (dpt) and fixed in buffer formalin 10%. Fixed tissues were dehydrated in graded alcohols series then cleared in xylene and embedded in paraffin wax, sections (at 3- 4 µm thickness) were stained with haematoxylin and eosin (H&E) following

routine procedures for histopathological investigation by light microscopy (Bancroft and Layton, 2013).

Results

Isolation and identification of E. coli

One hundred broiler samples were cultured, and 87% of the isolates were identified as *E. coli* by giving pink and green metallic sheen colonies on MacConkey's and Eosin Methylene Blue (EMB) agar, respectively. Biochemically, Indole was +ve, yellow slant, and yellow butt with gases on T.S.I, negative and positive results for Kosar citrate and catalase tests, correspondingly.

Detection of colistin resistant E. coli

E. coli isolates were 97.7% resistant to colistin, as indicated by their ability to grow on universal culture media, giving green metallic sheen colonies, and changing the color of Rapid Polymyxin Nordman test from orange to yellow colour.

Molecular detection of E. coli and mcr-1 gene

Isolates were positive for both *pho*A gene, which are characteristic of *E. coli*, and *mcr-1* gene, giving amplicon sizes at 720bp (Fig. 1) and 320bp (Fig. 2), respectively.

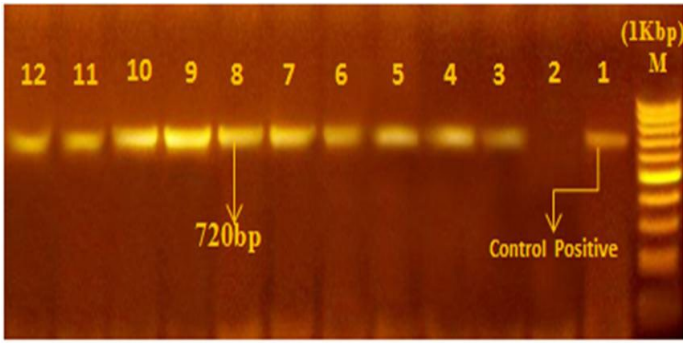

Fig. 1. DNA Ladder 1Kbp (M), lane 3-12 demonstrating *pho*A gene for *E. coli* Lane 1 (+ve control) giving amplicon size at 720bp and lane 2 gave no band (–ve result).

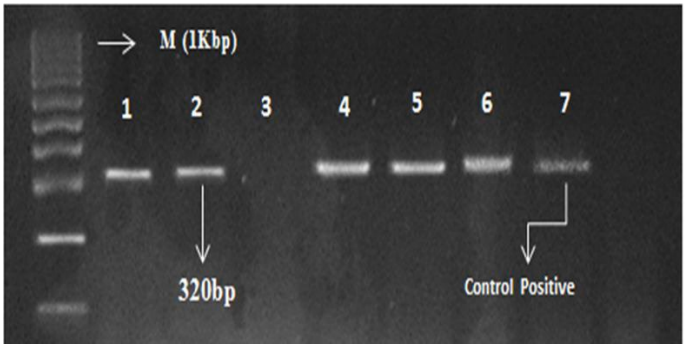

Fig. 2. DNA Ladder 1Kbp (M), Lane no. 1, 2, 4,5, and 6 representing *mcr-1* gene of colistin resistant *E. coli* isolates Lane 7 (+ve control) targeted band 320bp lane 3 gave no band (–ve result).

Serological identification of E. coli isolates with and without mcr-1gene

Findings of the experimental study

General signs were detected in both groups (1, 2) during 72 h post infection, which include loss of appetite, dullness, ophthalmitis, and diarrhea, mortality rate was 30% in group 1 (O91), and 16% in the other infected group (O78). The morbid anatomical picture revealed congestion of the liver and catarrhal to severe hemorrhagic enteritis. In the advanced stage of infection (after 72 h) necrosis and congestion in the liver, fibrinous perihepatitis, pericarditis, and enteritis ranging from mild to severe form were observed in both groups 1 and 2 that persisted to the end of this study (Fig. 3). Air sacculitis and mild to severe pneumonia were observed only in group 2, compared with group 3 (control -ve) that exhibited normal signs and postmortem until the end of the experiment.

The efficacy of neomycin clinically in both groups 4 and 5 revealed good prognosis, reached 80% perceived improvement by the absence of mortalities and fibrinous bi-layer in the internal organs (liver and heart) in group 4(O91 with *mcr-1*), while in group 5 (O78 without *mcr-1*), the effectiveness was 60% as there was moderate congestion in the liver and mild enteritis in some cases.

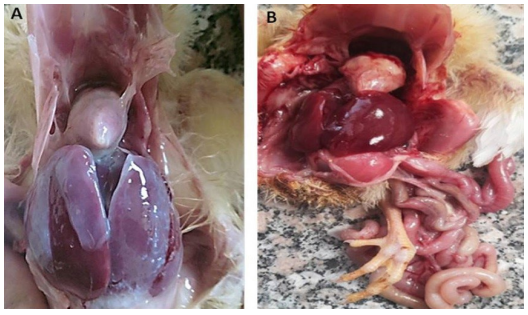

Fig. 3. (A) showing fibrinous pericarditis and perihepatitis (O91). (B) Fibrinous pericarditis, slight perihepatitis, congestion in liver and sever enteritis (O78).

Histopathological examination

Microscopic pictures of all healthy control negative chickens (group 3) had normal histological structure of heart and liver Figures 4, 5 (G-H). On the other hand, at 3 days post infection heart of chickens group 1(*E. coli* O91) showed mild sero-fibrinous pericarditis; sever degeneration and necrosis with inflammatory cell infiltrations of cardiac muscle (Fig. 4A). While, chickens of group 2 (*E. coli* O78) revealed fibrinous pericarditis associated with myocarditis (Fig. 4B). At 8 days post infection without neomycin treatment chicken heart of group 1(O91) showed marked fibrinous pericarditis characterized by thickening of pericardium with fibrinous exudates, congested blood vessels and myocarditis (Fig. 4C). Also, chicken of group 2 (O78) had fibrinous pericarditis expanded by multi-focal infiltrates of inflammatory cells (heterophils, macrophages and lymphocytes) that extended to myocardium with diffuse inflammatory cell infiltrations (Fig. 4D). At 5 days post neomycin treatment birds of group 4 (O91) revealed remarkable improvements and the pericardium appeared with normal histological structure while cardiac muscle only had mild congestion (Fig. 4E) compared with group 5 (O78) neomycin had no effect where this group still had marked fibrinous pericarditis and myocarditis (Fig. 4F).

Liver of group 1(O91) birds at 3dpi showed mild perihepatitis, sub-capsular congested dilated sinusoids with erythrocytes and inflammatory cells in addition to hepatocellular degeneration and coagulative necrosis (Fig. 5A). In group 2 (O78) portal edema observed in some cases and characterized by sero fibrinous exudate associated with inflammatory cells aggregation and surrounded by diffuse hepatocellular vacuolar degeneration (Fig. 5B). At 8dpi without neomycin treatment birds of group 1(O91) showed thickening of perihepatic capsule with fibrinous exudates and multifocal inflammatory cell infiltrations, hepatocellular degeneration, and coagulative necrosis (Fig. 5C). In group 2 (O78) perihepatitis characterized by extensive widening by fibrinous exudate with heterophils and lymphocytes infiltrations, vacuolar hepatocellular degeneration, and congested sinusoids (Fig. 5D). At 5dpt with neomycin group 4 (O91) there was an improvement in hepatic parenchymal and capsular lesions except minimal focal inflammatory cells aggregations were recorded (Fig. 5E). However, group 5 (O78) had no improvement in fibrinous perihepatitis or in hepatocellular degeneration and necrosis were noticed (Fig. 5F).

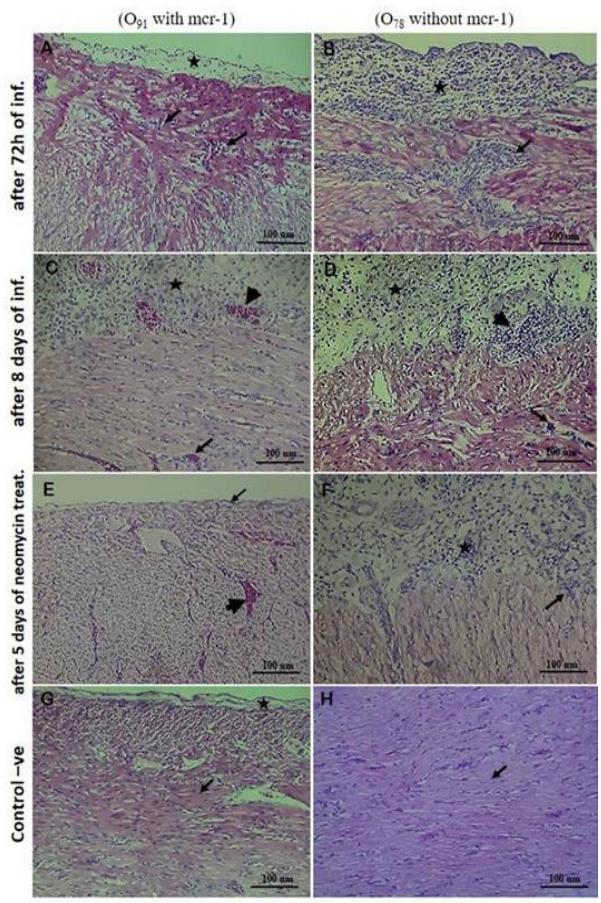

Fig. 4. Photomicrographs of chicks' heart stained with H&E stain showing (A-B) *E. coli* (O91, O78) at 3 dpi untreated (A) mild sero-fibrinous pericarditis (star), myocardium coagulative necrosis infiltrated with inflammatory leukocytes (arrows). (B) Fibrinous pericarditis (star), diffuse inflammatory cells infiltration (arrow) and myocarditis. (C-D) *E. coli* (O91, O78) at 8dpi untreated (C) thick fibrinous pericarditis (star) with extravasated erythrocytes (arrowhead) inter myocardial congestion and hemorrhages (arrow). (D) Pronounced fibrinous pericarditis (star) with lymphocytes and heterophils aggregation (arrowhead), degeneration of myocardium fibers with inflammatory cells infiltration (arrow). (E-F) *E. coli* infected & 5dp neomycin treatment (E) Nearly normal pericardium (arrow), congestion of myocardium blood vessels (arrowhead). (F) Marked fibrinous pericarditis (star) inflammatory cells infiltration (arrow). Control –ve (G-H) normal pericardium (star) and myocardium muscle fibers (arrow). Scale bar = 100 µm

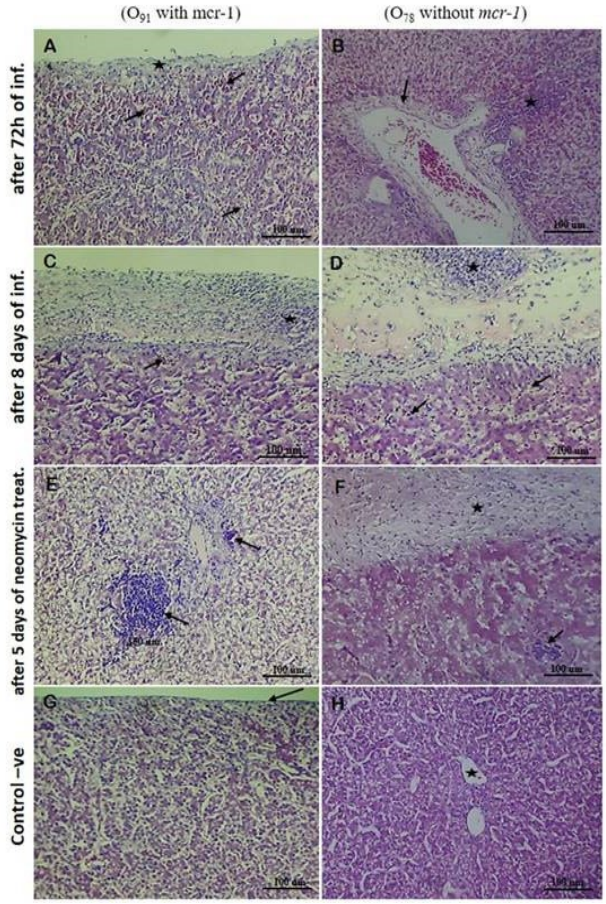

Fig. 5. Photomicrographs of chicks' liver stained with H&E stain showing (A-B) *E. coli* (O91, O78) at 3 dpi untreated (A) mild fibrinous perihepatitis (star), dilated sinusoid with erythrocytes and inflammatory leukocytes (arrows). (B) Congested blood vessel, periportal edema with sero-fibrinous exudate (arrow) associated with focal heterophils and lymphocytes aggregations (star). (C-D) *E. coli* (O91, O78) at 8dpi untreated (C) fibrinous perihepatitis with inflammatory cells infiltration (star), congested sinusoids (arrow). (D) Pronounced fibrinous perihepatitis with excessive fibrinous exudate and inflammatory cells infiltration (star), vacuolar hepatic degeneration and dilated congested sinusoids (arrows). (E-F) *E. coli* infected & 5dp neomycin treat. (E) Focal heterophils and lymphocytes aggregation (arrows). (F) Fibrinous perihepatitis (star), hepatocytes vacuolar degeneration and focal leukocytes aggregation (arrow). (G-H) Control –ve and (G) normal perihepatic capsule (arrow). (H) Normal hepatic blood vessel (star). Scale bar = 100 µm

Discussion

Colibacillosis is one of the most devastating diseases that threaten the poultry industry worldwide and public health. In this study, a high prevalence of avian colibacillosis, among broiler chicken farms was 87%; this was nearly in agreement with El-Sukhon *et al.* (2002) and Abd El Aziz *et al.* (2007) who recorded that prevalence was at 88.2% and 90%, respectively. On the other hand, a low prevalence of 43.1%, and 41.5% was recorded by Roshdy *et al.* (2012) and Radwan *et al.* (2014), respectively. These variations may be due to the differences in strains pathogenicity, virulence, and immunological status of birds, stress factors, and seasons.

The plasmid-mediated colistin resistance *mcr-1* gene identification is a further basis of worry due to the increasing observation of polymyxin resistance as a high rate of colistin resistance that was observed among our *E. coli* isolates (97.7%) through the phenotypic methods (super polymyxin media and rapid polymyxin NP tests), indicating the high specificity and sensitivity of super polymyxin NP test which was similar to studies done by Nordmann *et al.* (2016b) and Dalmolin *et al* . (2019). Moreover, the study revealed that the mechanism of colistin resistance in *E. coli* isolates was plasmid, not chromosomal, and this could refer to the risk of horizontal gene transfer.

Concerning the experimental study, evaluating of the existence of the *mcr-1* gene on the sensitivity of neomycin (hydrophobic antibiotic), revealed that chickens were active with normal appearance after 72h post treatment, there was nearly 80% gross enhancement indicated by the absence of mortalities and fibrinous bi-layer in the internal organs (liver and heart) in Group 4 (O91 with *mcr-1*), whereas in Group 5 (O78 without *mcr-1*), the efficacy was 60% with moderate congestion in the liver and mild enteritis in some cases, and this improvement in the drug action may be due to lipopolysaccharides modification because of *mcr-1* gene existence, this result agrees with Li *et al.* (2020) who indicated that the deletion of *mcr-1* increases resistance to hydrophobic antibiotics as *mcr-1* confers colistin resistance through the addition of cationic phosphoethanolamine (pEtN) to phosphate groups on the lipid A component of LPS, which reduces the net anionic charge of the cell surface, and suggested that the expression of *mcr-1* resulted in the alteration of LPS through disrupted organization of the LPS-phospholipid bi-layer, modified its permeability, and therefore increased the resistance to hydrophobic antibiotics. On the other hand, the infected Groups 1 (O91) and 2(O78) revealed typical signs and postmortem lesions of colibacillosis, with mortality rates reached 30% and 16%, respectively, that could be attributed to the variable virulence of each *E. coli* strain and similar lesions as reported by Ameh *et al.* (2011).This indicates the link between the *mcr-1* expression and the increased sensitivity to hydrophobic antibiotics, and this comes in line with ((Li *et al.*, 2020), who concluded that the involvement of *mcr-1* defends the bacteria against colistin, but modulates the membrane permeability and decreases resistance to hydrophobic antibiotics, which clarify the sense of balance between bacterial persistence and the *mcr-1* expression.

In the present study, gross lesions of chickens experimentally infected with *E. coli* (O91,O78), which included fibrinous pericarditis, myocarditis, fibrinous perihepatitis, vacuolar degeneration and necrosis of hepatic tissue, were similar to those previously described by EL-Sawah, *et al.* (2018) and Abd El-Mawgoud *et al.* (2020) who reported the same lesions in heart, liver and air sacs of broiler chickens challenged with APEC O78. Upon histopathological examination of chickens of group 4 (O91) it was observed that neomycin antibiotic had shown noticeable efficacy by reducing pathological changes of *E. coli* O91 with the *mcr-1* gene indicating that neomycin as a hydrophobic antibiotic (in the presence of the *mcr-1* gene) could cross altered bacterial outer membrane (OM) reaching intra-cellular target. On the other hand, when compared with its efficacy on *E. coli* (O78) without the *mcr-1*, in group 5, neomycin had no efficacy on the bacteria and its pathological lesions remained.

Conclusion

This study has detected the increased rate of the *mcr-1* gene existence in field *E. coli* strains isolated from broiler chickens in Assiut governorate, Egypt, and indicated, as a first report, the in vivo increased sensitivity of neomycin as a hydrophobic antibiotic in the presence of the *mcr-1* gene.

Conflict of interest

The authors declare that they have no conflict of interest.

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