BMC Microbiology

RESEARCH

Open Access

Potential of ZnO nanoparticles for multi-drug resistant *Escherichia coli* having CRISPR-Cas from poultry market in Lahore

Muhammad Abu Bakr Shabbir[1], Muqaddas Shamim[1], Adnan Hassan Tahir[2], Adeel Sattar[3], Wu Qin[4], Waqas Ahmad[5], Waqas Ahmad[6], Farid Ahmed Khan[1] and Muhammad Adnan Ashraf[1*]

Abstract

Background and objectives Apart from known factors such as irrational use of antibiotics and horizontal gene transfer, it is now reported that clustered regularly interspaced short palindromic repeats (CRISPR) are also associated with increased antimicrobial resistance. Hence, it is critical to explore alternatives to antibiotics to control economic losses. Therefore, the present study aimed to determine not only the association of CRISPR-Cas system with antibiotic resistance but also the potential of Zinc Oxide nanoparticles (ZnO-NPs) for avian pathogenic *Escherichia coli* (APEC) isolated from poultry market Lahore.

Materials and methods Samples ($n=100$) were collected from live bird markets of Lahore, and isolates were confirmed as *Escherichia coli* (*E. coli*) using the Remel One fast kit, and APEC was identified using PCR. The antibiotic resistance pattern in APEC was determined using the minimum inhibitory concentration (MIC), followed by genotypic confirmation of antibiotic-resistant genes using the PCR. The CRISPR-Cas system was also identified in multidrug-resistant (MDR) isolates, and its association with antibiotics was determined using qRT-PCR. The potential of ZnO-NPs was evaluated for multidrug-resistant (MDR) isolates by MIC.

Results All isolates of APEC were resistant to nalidixic acid, whereas 95% were resistant to chloramphenicol and 89% were resistant to streptomycin. Nineteen MDR APEC were found in the present study and the CRISPR-Cas system was detected in all of these MDR isolates. In addition, an increased expression of CRISPR-related genes was observed in the standard strain and MDR isolates of APEC. ZnO-NPs inhibited the growth of resistant isolates.

Conclusions The findings showed the presence of the CRISPR-Cas system in MDR strains of APEC, along with the potential of ZnO-NPs for a possible solution to proceed. This highlights the importance of regulating antimicrobial resistance in poultry to reduce potential health consequences.

Keywords CRISPR-Cas, *Cas-3*, APEC, Chicken, Antimicrobial resistance, Nanoparticles, ZnO-NPs

*Correspondence:
Muhammad Adnan Ashraf
adnan.ashraf@uvas.edu.pk
Full list of author information is available at the end of the article

Introduction

The poultry industry has developed rapidly over the last three decades, with an impressive growth rate of over 8% [1]. Commercial poultry in Pakistan contributes significantly to the livestock sector of the country's GDP, adding 1,809,000 tonnes of meat and 21,285 million eggs in FY 2021 [2]. The rise of poultry production is the need of the hour for food security as the industry is always vulnerable to disease-causing pathogens. APEC is the causative agent of colibacillosis in chickens, which is characterized by its ability to cause severe diseases in birds, such as respiratory infections, septicemia, and cellulitis [3]. The disease has resulted in significant financial losses due to decreased weight gain, disease prevention and control expenses, mortality, and carcass condemnations [4]. In a farm, APEC strains cause a 2–4% decrease in egg output and a 3–4% increase in bird mortality [5].

Many researchers have characterized different criteria for APEC identification. Phenotypically, these isolates display diverse traits, including the ability to hemagglutinate red blood cells, resist multiple antibiotics, and survive under stressful conditions like high temperature and low pH. Genotypically, APEC isolates have been established to be identified by one to multiple specific virulence-associated genes, such as *iss* (increased serum survival) and tsh (temperature-sensitive hemagglutinin) by Zhao et al. [6], and *iroN* (iron-responsive outer membrane protein gene), *ompT* (outer membrane protein A gene) *hlyF* (hemolysin F gene), *iss*, *iutA* (ferric aerobactin receptor gene) by Johnson et al. [7] and multiple combinations of three genes by Van der Westhuizen and Bragg [8]. The presence of these genes relates to the pathogenic potential of APEC isolates, but the criteria are not final.

Antibiotics such as tetracycline, fluoroquinolones, sulfonamides, and aminoglycosides are commonly used in poultry flocks to control colibacillosis. For many years, farmers in underdeveloped nations have used antibiotics for disease prevention and growth enhancement [9]. Resistance of *E. coli* has been increased with time, due to improper usage, or irrational usage of antibiotics [10] along with some other factors such as CRISPR-Cas system.

The CRISPR-Cas system is an adaptive immune system of bacteria that protects it from invaders such as bacteriophages and foreign genetic material [11]. This system is divided into three types: type I, II, and III [12]. The three subtypes of the CRISPR-Cas system are type I, type II, and type III. This classification was created based on the distinctive genes each category had *cas10*, *cas9*, and *cas 3* genes present in Type I, Type II, and Type III, respectively. Furthermore, its potential as a gene editing tool has various applications, including gene knockout, sickle cell therapy, and CRISPR-modified crops [13]. On an alternative aspect, the CRISPR-Cas system has been reported to have improved the integrity of the bacterial membrane and regulated its permeability [14], which may play a role in antimicrobial resistance [15]. Alternatively, it may possibly play a role in making bacteria immune to antibiotics that target membranes. However, there is a scarcity of information regarding the relationship between the CRISPR-Cas system and antibiotic resistance of APEC isolates.

With the rise in antibiotic resistance, alternative therapeutics are being explored. Nanoparticles (NPs), particularly ZnO-NPs, have shown potential for antibacterial activity and have been investigated for their antimicrobial properties in applications such as food packaging and antibiotic agents in animal feed [16]. Zinc is a crucial trace element for poultry, which is necessary for its growth and production. However, the zinc content in the raw materials used for poultry feed is often inadequate, necessitating zinc supplementation. Traditionally, inorganic zinc supplements have been used, but these have low bioavailability [17]. However, excessive use of dietary ZnO can disrupt the balance of other trace elements available to poultry. Alternatively, ZnO-NPs may offer improved bioavailability and absorption efficiency [18]. Therefore, ZnO-NPs have been justified for their dual use in the poultry industry to enhance zinc bioavailability and provide antibacterial benefits.

Keeping in view the possible association of CRISPR-Cas system with antimicrobial resistance and the potential of ZnO-NPs in antibacterial activity, the present study is designed to study CRISPR-Cas system in APEC isolates from Lahore, Pakistan, followed by the in-vitro study of ZnO-NPs on the bacterial isolates.

Materials and methods

Sample collection and bacterial isolation

A100 bird cloacal swab samples were collected from the commercial poultry market in Lahore and transported to the Bacteriology laboratory in the Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan. The sample size was calculated using the Epitools online sample size calculator (https://epitools.ausvet.com.au/). The bacteria were isolated through conventional methods [10]. The isolates were confirmed biochemically by using the Remel RapID One rapid kit, following the manufacturer's instructions. The generated color code was then confirmed using ERIC© software [19].

Identification of APEC through PCR

A commercially available GeneJET Genomic DNA Purification Kit (*Thermo Scientific*, Lithuania) was used to extract the DNA from all biochemically confirmed

isolates. The molecular identification of APEC was carried out using PCR with specific primers which were reported previously (Table 1). Conventional PCR was used to amplify the *iroN*, *ompT*, *hlyF*, *iss*, and *iutA* genes to confirm the presence of APEC isolates [7]. For the reaction mixture, 9 µL of nuclease-free water, 12.5 µL of 2x PCR Taq Plus MasterMix (ABM, Richmond, BC, Canada), 2 µL each of forward and reverse primers, and 1.5 µL of DNA template were used and amplified in Veriti 96-Well Thermal Cycler (Applied Biosystems™, USA). The PCR amplicons were separated by electrophoresis on a 1.5% agarose gel for 30 min at 110 volts. Subsequently, the gel was visualized using a gel documentation system (Omega Flour plus system, Aplegen Inc, California, USA) along with the GeneRuler™ 100 bp plus DNA ladder as a size reference.

Antibiotic susceptibility test

The MICs of various antibiotics including nalidixic acid (NAL), chloramphenicol (CHL), streptomycin (STR), gentamycin (GEN), ciprofloxacin (CIP), and tetracycline (TET) were studied using the CLSI standard broth dilution method [25], and were categorized as either sensitive or resistant. Serial dilutions of antimicrobials were prepared with concentrations ranging from 1024 mg/ml to 0.125 mg/ml. The bacterial concentration was adjusted to 10^8 CFU/ml using the 0.5 McFarland's standard. These dilutions were then used to estimate the MIC. The optical density of the tubes was observed both before and after incubation to verify the MIC value.

Table 1 Primers used to identify virulent genes, antibiotic-resistant genes, and CRISPR-Cas genes of avian pathogenic *E. coli* isolated from poultry

Gene	Specific primers	Product size	References
iss	F-CAGCAACCCGAACCACTTGATG R- AGCATTGCCAGAGCGGCAGAA	323	[20]
iroN	F-AATCCGGCAAAGAGACGAACC GCCT R- GTTCGGGCAACCCCTGCTTTG ACTTT	553	[21]
ompT	F-TCATCCCGGAAGCCTCCCTCACTA CTAT R-TAGCGTTTGCTGCACTGGCTTCTG ATAC	496	[21]
hlyF	F-GGCCACAGTCGTTTAGGGTGC TTACC R-GGCGGTTTAGGCATTCCGATA CTCAG	450	[22]
iutA	GGCTGGACATCATGGGAACTGG CGT CGGGAACGGGTAGAATCG	302	[7]
gyrA	F-AGCAGGACGAACGTATCACT R-AGTCATCTACCTGTACCGCG	498	This study
catA	F-AGTTGCTCAATGTACCTATAACC R-TTGTAATTCATTAAGCATTCTGCC	547	[23]
aac	F-TTGCGATGCTCTATGAGTGGCTA R- CTCGAATGCCTGGCGTGTTT	482	[24]
cas1	F- CAACAGGCTGTGCATCTTCA R- ACCTGGCTTCCCCTTAATCC	865	This study
cas2	F- CCATCCAAATCTACCGGGGT R- AGTATGTTGGTCGTGGTCACT	251	This study
cas3	F- TGGGATTTGCAGGGATGACT R- TGCTACCAACGGCTAAAGGA	916	This study
*Cas 3**	F- GACGGATACTTGTCGCAACC R-CTCATGTCGTCCGTAACCCT	206	This study

Antibiotic-resistant genes detection

The antibiotic-resistant genes such as *gyrA*, *catA*, and *aac* were detected by PCR, as described previously [23]. A PCR mixture (25µL) was created using nuclease-free water (9µL), 2x PCR Taq plus MasterMix (12.5µL), forward and reverse primers (2µL each), and DNA template (1.5µL).

Detection of CRISPR genes

The CRISPR genes were detected in confirmed MDR isolates of APEC through PCR, and the specific primers of *cas1*, *cas2*, and *cas3* are mentioned in Table 1. The amplified PCR products were electrophoresed and analyzed using an Omega Fluor Plus Systems gel documentation system (Omega Flour Plus system, Aplegen Inc, California, USA).

CRISPR gene expression after exposure to antimicrobial by qRT-PCR

The qRT-PCR was performed to identify CRISPR gene expression in antibiotic-resistant APEC after exposure to antibiotics. One MDR APEC isolate, and its standard strain was exposed to six antibiotics. The RNeasy Mini Kit (Tiagen Biotech, Beijing, PR China) was used to extract bacterial RNA from each sample. A Revert Aid First-Strand cDNA Kit (Thermo Scientific, USA) was used to make cDNA. The CFX96 real-time PCR thermocycler (Bio-Rad, Singapore) was used for amplification. Pre-incubation at 95 °C for 3 min was followed by 45 cycles of 10 s at 95 °C and 40 s at 52 °C in the cycling conditions for amplification. The specific primers used were *cas3*∗F (GACGGATACTTGTCGCAACC) and *cas3*∗R (CTCATGTCGTCCGTAACCCT) (Table 1). The experiment was repeated three times to calculate the mean fold change. The housekeeping gene *rpsL* was used as a control.

Antibacterial activity of ZnO-NPs

ZnO-NPs were used in a previous study [26]. The MICs of ZnO-NPs were studied using the CLSI standard broth dilution method as performed above for antibiotics [25]. Serial dilutions of ZnO-NPs were prepared with concentrations ranging from 512 mg/ml to 0.125 mg/ml.

Statistical analysis

The statistical analysis was conducted using IBM Corp.'s (Armonk, NY, USA) SPSS software, version 25.0. Using a two-way Analysis of Variance (ANOVA) and Tukey's post hoc test, the zone of inhibition along with relative fold expression of the *cas 3* gene in *E. coli* ATTC 25922 and *E. coli* local isolate against different antibiotics was compared. The relationship of the *cas 3* gene and the zone of inhibition of various antibiotics was checked by Pearson's correlation coefficient. GraphPad Prism 9.5.0 (San Diego, CA, USA) was used to generate a graphical depiction of the comparison.

Results

Identification of *E. coli* isolates

The collected samples were grown and processed using conventional bacteriological techniques. After identification by Gram staining (pink colored rods in microscope) and Remel RapID One kit; 60% (60/100) isolates were confirmed as *E. coli*.

Confirmed APEC isolates

APEC was identified by amplification of all isolates identified as *E. coli* by using specific primers of *iroN*, *ompT*, *hlyF*, *iss*, and *iutA* genes. The 50 isolates were identified as APEC as all these isolates showed specific amplicons for these genes. The results of genus-specific amplification were observed on 1.5% agarose gel. The gel was seen after electrophoresis using UV light in the gel documentation system, as shown in Fig. 1.

Antimicrobial susceptibility patterns

The MIC method was used to check the susceptibility of all 50 confirmed isolates of APEC (Fig. 2a). The

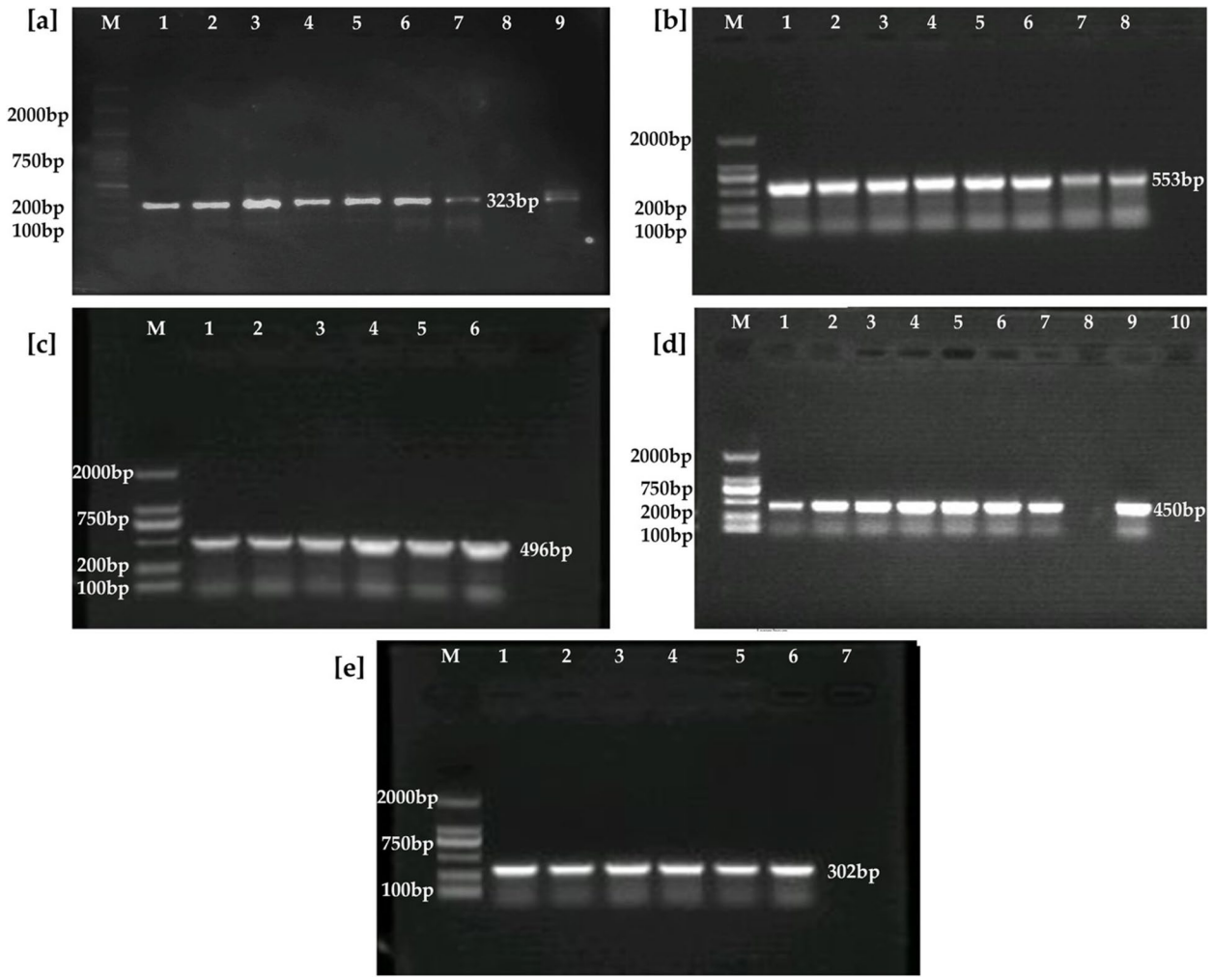

Fig. 1 Detection of APEC isolates through PCR: (**a**) the presence of the *iss* gene, (**b**) the presence of the *iroN* gene, and (**c**) the presence of the *ompT* gene [**d**] the presence of *hlyF* [**e**] the presence of *iutA*. M indicates the molecular marker. The numeric characters represent the sequential number of APEC isolates

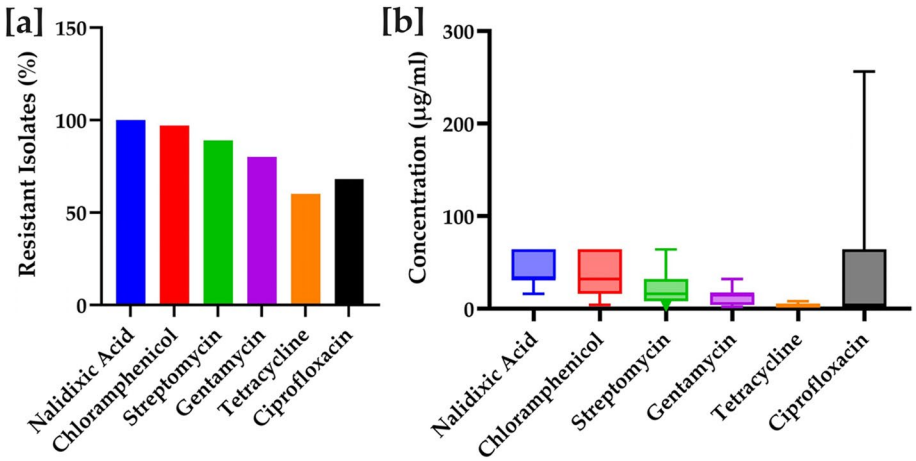

Fig. 2 Graphical presentation of antibiotic susceptibility of APEC isolates: (**a**) the percentage (%) resistance of APEC isolates against different antibiotic agents (**b**) the box and whisker plot of MIC of Nalidixic acid, chloramphenicol, streptomycin, gentamicin, ciprofloxacin, tetracycline against APEC isolates

following antibiotics NAL, CHL, STR, GEN, CIP, and TET were used against APEC. The isolates of APEC showed high levels of resistance to NAL (100%), CHL (95%), STR (89%), and GEN (80%), followed by intermediate levels of resistance to CIP (68%) and TET (60%) in the present study. The MIC values of various antibiotics against the APEC isolates in the present study showed that NAL and CHL had a median MIC of 32 µg/mL. STR revealed a median MIC of 16 µg/mL (IQR; 8–32 µg/mL). While GEN, TET, and CIP also had a median MIC of 16 µg/mL (IQR; 4–16 µg/mL), 4 µg/mL (IQR; 2–4 µg/mL), and 4 µg/mL (IQR; 0.5000–64 µg/mL), respectively. In contrast, ZnO-NPs revealed a median MIC of 64 mg/mL (IQR; 32–128 mg/mL).

Antibiotic-resistant genes detection

PCR was used to identify drug resistance genes (*gyrA*, *catA*, and *aac*) in confirmed APEC isolates that showed resistance in an antibiotic susceptibility test. Out of 50 isolates, 19 were MDR isolates and contained all 3 antibiotic resistance genes, and the results were analyzed as shown in Fig. 3. At the same time, non-MDR isolates contained 1 or 2 genes of antibiotic resistance.

Detection of CRISPR-Cas system

The CRISPR-Cas system was confirmed in MDR isolates of APEC. The CRISPR was detected by screening *cas1*, *cas2*, and *cas3* genes through PCR, and amplification results were presented in Fig. 4.

Expression of CRISPR-Cas gene in the standard strain and APEC Isolate

The correlation between the CRISPR-Cas system and antibiotic resistance was confirmed using qRT-PCR. The expression of the *cas3* gene increased in the MDR of APEC isolate and the *E. coli* ATCC 25922 strain, as shown in Fig. 5. This high expression may be due to the CRISPR-Cas system, which controls several genes that maintain membrane integrity and battle various stresses like antibiotic resistance.

The relative *cas3* gene expression in *E. coli* ATCC 25922 and local isolate to antibiotics was compared using two-way ANOVA. The local isolate showed a statistically significant increase ($p < 0.05$) in the fold expression level when exposed to chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, streptomycin, tetracycline, and zinc oxide in comparison to the ATCC strain. The relative expression of the *cas3* gene varied from 1.58 to 2.47 in the local isolate versus from 1.22 to 1.62 in the ATCC 25922 strain under different antimicrobial treatments. However, the least *cas3* expression was observed against ZnO-NPs in both ATCC 25922 (1.22 ± 0.03) and local isolate (1.58 ± 0.04). Whereas, the highest values were recorded against gentamycin (1.62 ± 0.02) and nalidixic acid (2.47 ± 0.04) in ATCC 25922 and local isolate, respectively (Table 2).

Antibacterial Activity of ZnO-NPs

ZnO-NPs revealed a median MIC of 64 mg/ml (IQR; 32-128 mg/ml) (Fig. 6a). The descriptive analysis of APEC isolates exposed to varying concentrations of ZnO-NPs

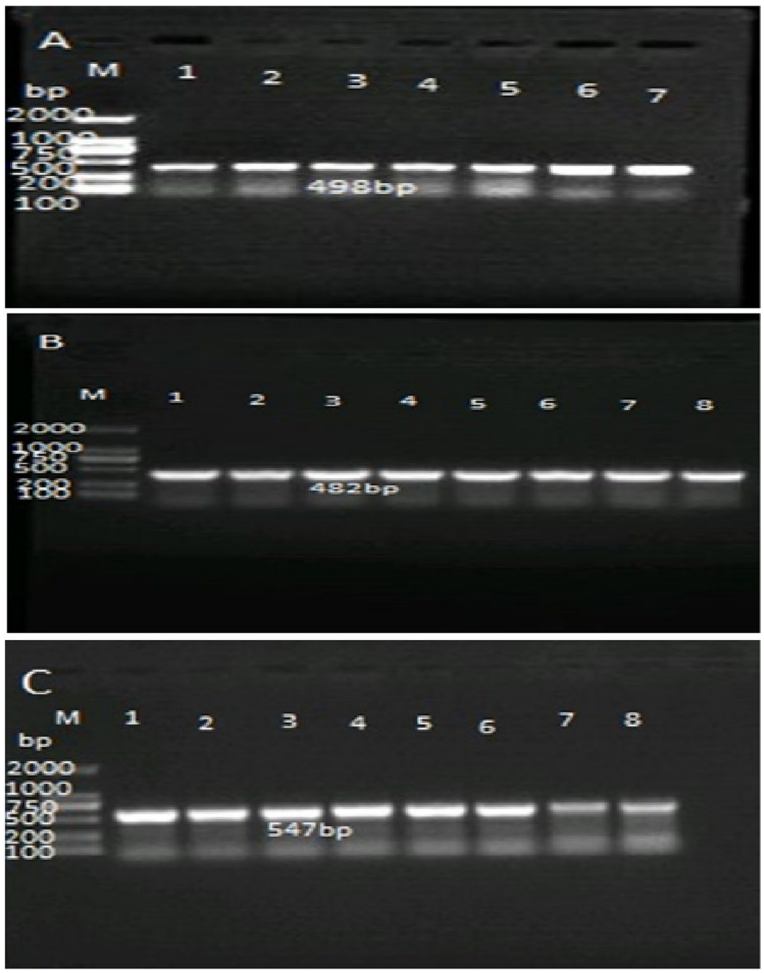

Fig. 3 Detection of antibiotic resistance genes through PCR: (**A**) the presence of the *gyrA* gene, (**B**) the presence of the *aac* gene, and (**C**) the presence of the *catA* gene. M indicates the molecular marker. The numeric characters represent the sequential number of APEC isolates

demonstrated that out of 50 isolates, 13 were inhibited (MIC) at a concentration of 32 mg/ml. Meanwhile, 22 isolates showed a MIC of 64 mg/ml. Additionally, eleven and four isolates revealed the MIC value of 128 mg/ml and 256 mg/ml, respectively (Fig. 6b).

Discussion

The CRISPR-Cas systems have played a significant role in the adaptive immune system against invading genetic material. These processes are also important in the survival and evolution of bacteria [12]. However, its contribution to antibiotic resistance hasn't been fully explored. The present study explored the possible association of the CRISPR system with antibiotic resistance in APEC isolates using phenotypic and genotypic approaches. Furthermore, ZnO-NPs is explored as an alternative therapeutic potential for MDR APEC strains.

The current study used genus-specific-specific PCR for five genes to detect the APEC. Multiple researchers have tried to establish a diagnostic approach for identifying APEC isolates on a molecular basis, including single to multiple virulence-associated genes. Foley et al. found *iss* gene to be sufficient for virulent *E. coli*, and the argument is supported by Johnson et al., who found it to be significantly higher in APEC than other isolates [20, 27]. Five genes which were more commonly suggested by multiple researchers, were identified in the present study as well. These include *iroN*, *ompT*, *hlyF*, *iss*, and *iutA* [7, 28]. Van der Westhuizen and Bragg [8] suggested 6 sets of multiplex PCRs, each including three genes for APEC identification which include (1) *iss*, *iucC* and *cvaC*; (2) *iroN*, *tsh*, and *yjj*; (3) *vat*, *kpsM*, and *sitA*; (4) *frz*, *sitD*, and *fimH*; (5) *ompT*, *iutA*, and *pstB*; (6) *sopB*, *uvrY*, and *hlyF* [8]. In contrast to our study, Gines et al. suggested eight important

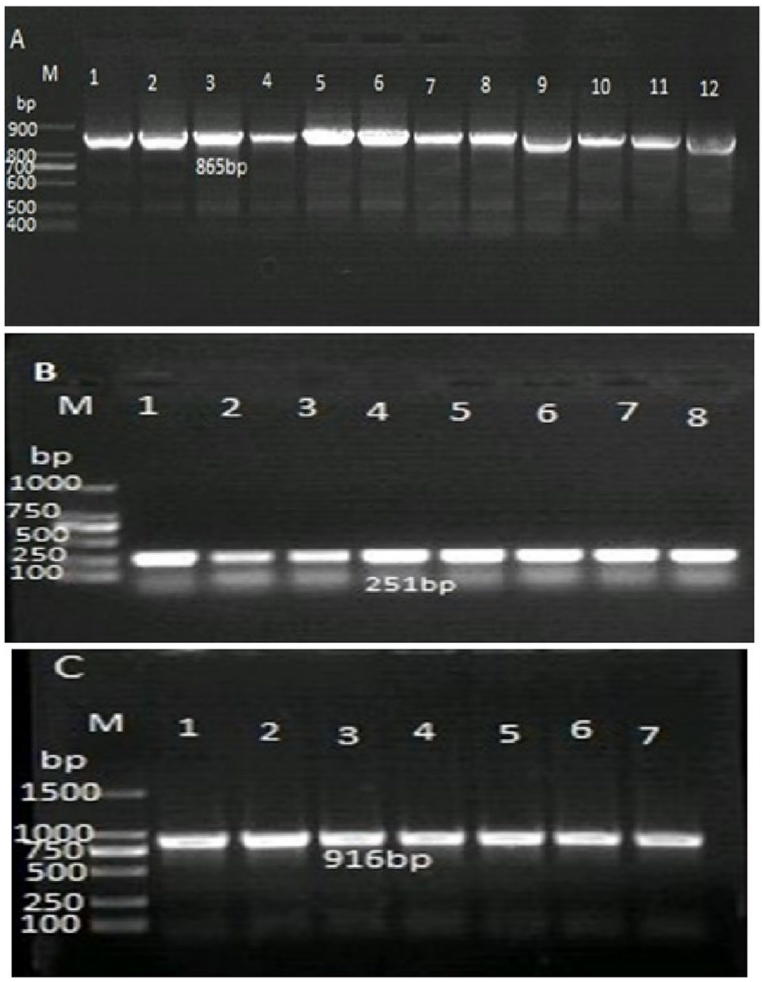

Fig. 4 Detection of the CRISPR-Cas system in MDR isolates of APEC: (**A**) the presence of the *cas1* gene, (**B**) the presence of the *cas2* gene and (**C**) the presence of the *cas3* gene. M indicates molecular marker. The numeric characters represent the sequential number of APEC isolates

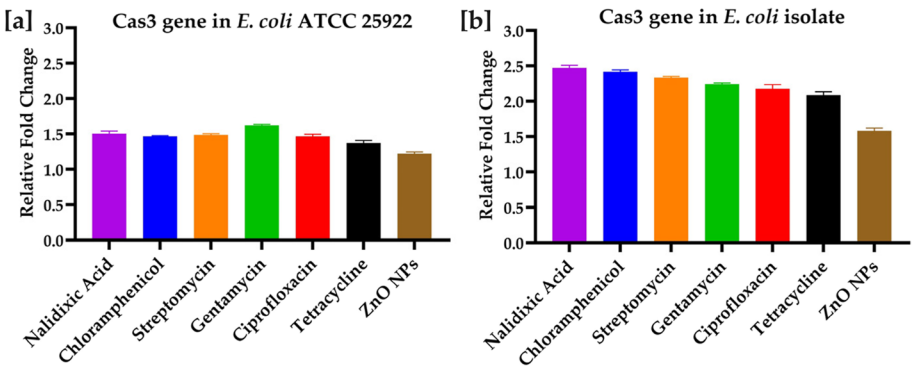

Fig. 5 Expression of *cas3* gene in APEC isolate and *E. coli* ATCC 25922 under the exposure of different antibiotics and ZnO-NPs: (**a**) shows the expression of the *cas3* gene in *E. coli* ATCC 25922 under the exposure of antibiotics and ZnO-NPs and (**b**) shows the expression of the *cas3* gene in *E. coli* isolate under the exposure of antibiotics and ZnO-NPs

Table 2 Comparison of *cas3* gene relative Fold expression in *E. coli* ATCC 25922 and local isolate when exposed to various antimicrobial agents

Antibiotic	Relative Fold Expression	
	***cas3* gene in *E. coli* ATCC 25,922**	***cas* gene in *E. coli* local isolate**
Chloramphenicol	1.47 ± 0.00882^{ef}	2.42 ± 0.0267^{ab}
Ciprofloxacin	1.47 ± 0.0285^{ef}	2.18 ± 0.0623^{cd}
Gentamycin	1.62 ± 0.0173^{e}	2.25 ± 0.0176^{bcd}
Nalidixic Acid	1.5 ± 0.0384^{ef}	2.47 ± 0.0379^{a}
Streptomycin	1.49 ± 0.0167^{ef}	2.34 ± 0.0173^{abc}
Tetracyclin	1.38 ± 0.0318^{fg}	2.09 ± 0.047^{d}
Zinc Oxide	1.22 ± 0.0265^{g}	1.58 ± 0.0404^{e}

Values expressed as mean (± S.E.M) having different superscripts differ significantly ($p < 0.05$)

genes for the identification, which include *irp-2*, *iutA*, *ompT*, *iss*, *iucD*, *astA*, *hlyF*, and *iroN* virulence genes [5].

In the current research, 60 isolates of *E. coli* from poultry were identified as APEC, and 50 (50%) isolates were identified. While there are researchers who find certain groups of genes to be essential to declare APEC strains, there were studies that found the percentage of certain genes to be higher than others. Similarly, Azam et al. [29] found 100% of isolates (75/75) to have *iss* gene while 92% (69/75) to have a *gad* gene in APEC isolates. Surprisingly, certain virulence genes were also found in commensal isolates. The criteria for declaring APEC is debatable. However, largely 3–5 sets of genes have been suggested for the identification of APEC strains. However, APEC isolates exhibited a significantly higher presence of minimal virulence predictors. Nevertheless, the identification of virulence genes was found to be a more reliable criteria as compared to typing based on Pulsed-field gel electrophoresis (PFGE) and Multilocus sequence typing (MLST) [5].

APEC causes colibacillosis, which is a serious infection that affects many aspects of commercially grown poultry and results in significant financial losses [28]. Antimicrobial drug use in food animals, particularly birds, resulted in the transmission of dangerous disease strains to humans through food [10]. The present study showed a high level of resistance to nalidixic acid (100%), chloramphenicol (95%), streptomycin (80%) and gentamicin (78%). The antibiotic resistance trend determined in this study depicts a potentially dangerous condition of antibiotic-resistant APEC strains in commercial poultry in Pakistan. All these isolates were multi-resistant, therefore, therapeutic success may have been compromised, causing a serious economic burden to the broiler industry. These findings correspond with previous studies that found the highest resistance to quinolones and aminoglycosides compared to other antibiotics [19, 30].

Interestingly, the expected intermediate resistance levels for ciprofloxacin and tetracycline are similarly consistent with earlier literature [28, 31]. Previous studies revealed a substantial increase in veterinary antibiotic import in Pakistan due to inappropriate use in poultry. Also, the irrational use of antibiotics induces selection pressure on microorganisms, resulting in new multidrug-resistant pathogens. Several studies have demonstrated that the *E. coli* population of broilers are a reservoir of antimicrobial resistance genes that may be transferred by mobile genetic elements to the community via the food chain [32]. Although efforts have been implemented to reduce the use of antimicrobials in the poultry industry, these are sometimes overused in food-producing animals, particularly in broiler farms [33].

In the present study, the CRISPR-Cas system in MDR of APEC was detected using specific primers for the different *cas* genes (*cas1*, *cas2*, *cas3*). All MDR isolates of APEC were positive for the *cas* genes-specific PCR, and the results were in accordance with those

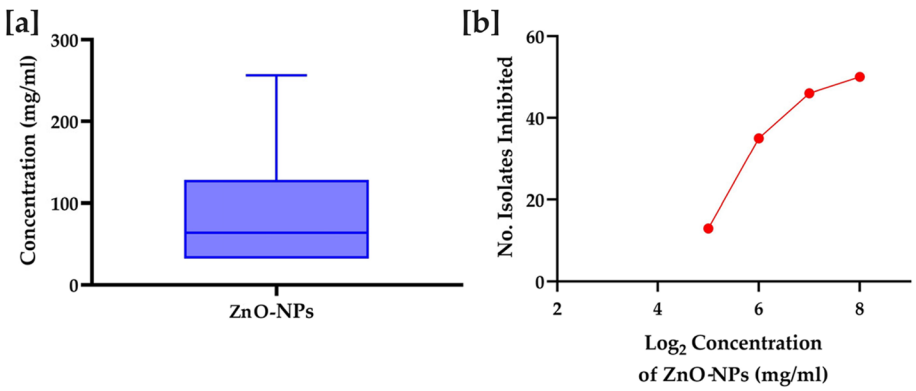

Fig. 6 Antibacterial activity of ZnO-NPs against APEC isolates: (**a**) the Box and Whisker plots showing the median (Interquartile range) of MIC of ZnO-NPs for the APEC isolates (**b**) Cumulative number of *E. coli* isolates inhibited by increasing log concentrations of ZnO nanoparticles (ZnO-NPs)

of previous studies [34]. The current study used qRT-PCR to assess the relationship between the CRISPR-Cas system and antibiotic resistance. CRISPR gene expression was higher in MDR isolates of APEC than in the reference strain, demonstrating that the system is associated with antibiotic resistance. As previously stated, this enhanced expression of CRISPR genes can be plausibly due to the regulation of numerous genes that play roles in membrane integrity, as the loss of CRISPR-Cas system in antibiotic-resistant species might have unforeseen regulatory impacts, resulting in changes to bacterial envelope structure and increased susceptibility to certain antibiotics [14]. Stress to bacterial envelope and host cell infection enhances CRISPR-Cas expression in bacteria [35, 36]. This suggests that CRISPR-Cas systems are activated by membrane stressors, leading to enhanced envelope integrity and increased resistance to these stressors, which may include antibiotics.

One of the objectives of the study was to test ZnO-NPs as an alternative therapeutic option. The results revealed varied susceptibility of the 50 APEC isolates with a median of less than 60 mg/ml having a range of 32–256 mg/ml. The results were similar to those of Yusof et al. [36], who found MIC to be 60 for *E. coli*. The antibacterial properties of ZnO-NPs are primarily due to the action of Zn2 + ions [37]. Findings showed that the smaller particle size of ZnO-NPs boosts their antibacterial efficacy because of their larger surface area to volume ratio, which increases surface reactivity and ion release [38]. Results were comparable to other studies where researchers found the action of ZnO-NPs promising for poultry pathogens [39, 40].

Limitation of the study

Among the few limitations, the study of virulence-associated genes commensal isolates along with pathogenic may have provided a better and more in-depth picture of APEC isolates identification. The current work provides initial evidence associating the CRISPR system to antibiotic resistance. The correlation between CRISPR-Cas system and antibiotic resistance was not explored in depth, necessitating further research to understand this relationship fully. Lastly, although innovative, the use of ZnO-NPs as an antimicrobial agent requires more extensive in vivo studies to evaluate its practical applicability and potential toxicity.

Conclusions

The antibiotic resistance profile of APEC isolated from poultry markets in Lahore, Pakistan, was examined in this study's conclusion. According to the findings, there was a high prevalence of APEC in chicken samples, and a sizable percentage of those samples had antibiotic resistance, particularly to NAL and CHL. Additionally, the CRISPR-Cas system was found in MDR isolates of APEC, providing evidence of a potential mechanism for the emergence of antibiotic resistance. Furthermore, ZnO-NPs have shown promising potential antibacterial activity in virulent isolates. These results underline the urgent need for efficient control methods to deal with the rising antimicrobial resistance in poultry and its possible health effects.

Abbreviations

CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPR-Cas	Clustered regularly interspaced short palindromic repeats-CRISPR-associated proteins
ZnO-NPs	Zinc oxide nanoparticles
APEC	Avian Pathogenic *Escherichia coli*
E. coli	*Escherichia coli*
PCR	Polymerase Chain Reaction
MIC	Minimum Inhibitory Concentration
MDR	Multi-drug resistance
qRT-PCR	Quantitative reverse-transcription PCR
MHA	Mueller Hinton Agar
ZOI	Zone of inhibition
ANOVA	Analysis of variance

Acknowledgements

Not applicable.

Authors' contributions

Conceptualization, M.A.B.S. and M.A.A.; methodology, M.S.; software, W.A5.; validation, A.S.; formal analysis, W.A5.; data curation, W.A6. and M.S. and W.Q.; writing—original draft preparation, M.A.B.S and F.A.K; writing review and editing, A.H.T and M.A.A; visualization, W.A6.; supervision, M.A.A. All authors have read and agreed to the published version of the manuscript.

Funding

This research received no external funding.

Availability of data and materials

All the data is provided within the manuscript.

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the guidelines of the Institutional Review Board of the University of Veterinary and Animal Sciences Lahore, Pakistan (Dr/290, dated 27 February 2023).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]Institute of Microbiology, Faculty of Veterinary Science, University of Veterinary and Animal Sciences, Lahore, Pakistan. [2]Department of Clinical Studies, Faculty of Veterinary and Animal Sciences, Pir Mehr Ali Shah-Arid Agriculture University, Rawalpindi, Pakistan. [3]Department of Pharmacology and Toxicology, Faculty of Biosciences, University of Veterinary and Animal Sciences, Lahore, Pakistan. [4]Key Laboratory of Applied Technology on Green-Eco-Healthy Animal Husbandry of Zhejiang Province, Zhejiang Provincial Engineering Laboratory for Animal Health Inspection & Internet Technology, Joint Laboratory for Animal Health Big Data Analytics, College of Animal Science and Technology, Zhejiang International Science and Technology Cooperation Base for Veterinary Medicine and Health Management,

China-Australia Joint Laboratory for Animal Health Big Data Analytics, College of Animal Science and Technology & College of Veterinary Medicine of Zhejiang A&F University, 311300 Hangzhou, China. [5]Department of Pathology, Faculty of Veterinary Sciences, University of Veterinary and Animal Sciences, Lahore, Pakistan. [6]Department of Clinical Sciences, University of Veterinary and Animal Sciences, Narowal Campus, Lahore, Pakistan.

Received: 14 May 2024 Accepted: 14 August 2024
Published online: 19 September 2024

References

1. GoP. Economic Survey of Pakistan 2020. Islamabad, Pakistan: Economic Advisory Wing, Finance Division; 2020.
2. GoP. Economic Survey of Pakistan 2020. Islamabad: Economic Advisory Wing, Finance Division; 2020.
3. Nolan LK, Barnes HJ, Vaillancourt JP, Abdul-Aziz T, Logue, CM. Colibacillosis. In: Swayne DE. editor. NJ, USA: Diseases of Poultry. John Wiley & Sons; 2013. p. 751–805.
4. Ronco T, Stegger M, Olsen RH, Sekse C, Nordstoga AB, Pohjanvirta T, Lilje B, Lyhs U, Andersen PS, Pedersen K. Spread of avian pathogenic *Escherichia coli* ST117 O78: H4 in nordic broiler production. BMC Genom. 2017;18:1–8.
5. Solà-Ginés M, Cameron-Veas K, Badiola I, Dolz R, Majó N, Dahbi G, Viso S, Mora A, Blanco J, Piedra-Carrasco N. Diversity of multi-drug resistant avian pathogenic *Escherichia coli* (APEC) causing outbreaks of colibacillosis in broilers during 2012 in Spain. PLoS ONE. 2015;10(11):e0143191.
6. Zhao S, Maurer JJ, Hubert S, De Villena JF, McDermott PF, Meng J, Ayers S, English L, White DG. Antimicrobial susceptibility and molecular characterization of avian pathogenic *Escherichia coli* isolates. Vet Microbiol. 2005;107(3–4):215–24.
7. Johnson TJ, Wannemuehler Y, Doetkott C, Johnson SJ, Rosenberger SC, Nolan LK. Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. J Clin Microbiol. 2008;46(12):3987–96.
8. Van der Westhuizen W, Bragg R. Multiplex polymerase chain reaction for screening avian pathogenic *Escherichia coli* for virulence genes. Avian Pathol. 2012;41(1):33–40.
9. Jalil A, Masood S, Ain Q, Andleeb S, Dudley EG, Adnan F. High resistance of fluoroquinolone and macrolide reported in avian pathogenic *Escherichia coli* isolates from the humid subtropical regions of Pakistan. J Global Antimicrob Resist. 2023;33:5–17.
10. Zainab L, Ibrar K, Sadiq A, Hamid A, Ullah M, Noor R. Extended spectrum beta lactamases-producing *Escherichia coli* in retail chicken meat from Khyber Pakhtunkhwa, Pakistan. Saudi J Biol Sci. 2022;29(6):103280.
11. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P. CRISPR provides acquired resistance against viruses in prokaryotes. Science. 2007;315(5819):1709–12.
12. Shabbir MAB, Hao H, Shabbir MZ, Hussain HI, Iqbal Z, Ahmed S, Sattar A, Iqbal M, Li J, Yuan Z. Survival and evolution of CRiSPR–Cas system in prokaryotes and its applications. Front Immunol. 2016;7:375.
13. Wang JY, Doudna JA. CRISPR technology: a decade of genome editing is only the beginning. Science. 2023;379(6629):eadd8643.
14. Sampson TR, Napier BA, Schroeder MR, Louwen R, Zhao J, Chin C-Y, Ratner HK, Llewellyn AC, Jones CL, Laroui H. A CRISPR-Cas system enhances envelope integrity mediating antibiotic resistance and inflammasome evasion. Proc Natl Acad Sci. 2014;111(30):11163–8.
15. Shabbir MA, Wu Q, Shabbir MZ, Sajid A, Ahmed S, Sattar A, Tang Y, Li J, Maan MK, Hao H. The CRISPR-cas system promotes antimicrobial resistance in *Campylobacter jejuni*. Future Microbiol. 2018;13(16):1757–74.
16. Ahmadi A, Ahmadi P, Ehsani A. Development of an active packaging system containing zinc oxide nanoparticles for the extension of chicken fillet shelf life. Food Sci Nutr. 2020;8(10):5461–73.
17. Azad S, Shariatmadari F, Torshizi M, Chiba L. Comparative effect of zinc concentration and sources on growth performance, accumulation in tissues, tibia status, mineral excretion and immunity of broiler chickens. Brazilian J Poult Sci. 2020;22(02):eRBCA–2019.
18. Zhao L, Xu Y-H, Akasaka T, Abe S, Komatsu N, Watari F, Chen X. Polyglycerol-coated nanodiamond as a macrophage-evading platform for selective drug delivery in cancer cells. Biomaterials. 2014;35(20):5393–406.
19. Azam M, Mohsin M, Saleemi MK. Virulence-associated genes and antimicrobial resistance among avian pathogenic *Escherichia coli* from colibacillosis affected broilers in Pakistan. Trop Anim Health Prod. 2019;51:1259–65.
20. Johnson TJ, Wannemuehler YM, Nolan LK. Evolution of the *iss* gene in *Escherichia coli*. Appl Environ Microbiol. 2008;74(8):2360–9.
21. Johnson TJ, Siek KE, Johnson SJ, Nolan LK. DNA sequence of a ColV plasmid and prevalence of selected plasmid-encoded virulence genes among avian *Escherichia coli* strains. J Bacteriol. 2006;188(2):745–58.
22. Morales C, Lee MD, Hofacre C, Maurer JJ. Detection of a novel virulence gene and a *Salmonella* virulence homologue among *Escherichia coli* isolated from broiler chickens. Foodbourne Pathog Dis. 2004;1(3):160–5.
23. Kim YB, Yoon MY, Ha JS, Seo KW, Noh EB, Son SH, Lee YJ. Molecular characterization of avian pathogenic Escherichia coli from broiler chickens with colibacillosis. Poult Sci. 2020;99(2):1088–95.
24. Liaqat Z, Khan I, Azam S, Anwar Y, Althubaiti EH, Maroof L. Isolation and molecular characterization of extended spectrum beta lactamase producing Escherichia coli from chicken meat in Pakistan. Plos One. 2022;17:e0269194.
25. CLSI. Performance standards for antimicrobial susceptibility testing. In.: Clinical and laboratory standard institute; 2020.
26. Lail N-u, Sattar A, Omer MO, Hafeez MA, Khalid AR, Mahmood S, Shabbir MA, Ahmed W, Aleem MT, Alouffi A. Biosynthesis and characterization of zinc oxide nanoparticles using *Nigella sativa* against coccidiosis in commercial poultry. Sci Rep. 2023;13(1):6568.
27. Foley SL, Horne SM, Giddings CW, Robinson M, Nolan LK. Iss from a virulent avian *Escherichia coli*. Avian Dis. 2000;44:185–91.
28. Usman S, Anjum A, Usman M, Imran M, Ali M, Moustafa M, Rehman M, Hussain T, Sarwar F, Azad A. Antibiotic resistance pattern and pathological features of avian pathogenic *Escherichia coli* O78: K80 in chickens. Brazilian J Biol. 2022;84:e257179.
29. Azam M, Mohsin M, Johnson TJ, Smith EA, Johnson A, Umair M, Saleemi MK. Genomic landscape of multi-drug resistant avian pathogenic *Escherichia coli* recovered from broilers. Vet Microbiol. 2020;247:108766.
30. Madni W, Mohsin M, Nawaz Z, Muzammil S, Zahoor M, Asif R. Molecular mechanism of antimicrobial co-resistance colistin (mcr-1) and ESBLs genes among *Escherichia coli* isolates from commercial chickens in Pakistan. Brazilian J Biol. 2023;84:e267494.
31. Kim MH, Kim S. Structures and functions of multi-tRNA synthetase complexes. The enzymes. Volume 48. Elsevier; 2020. pp. 149–73.
32. Johnson JR, Sannes MR, Croy C, Johnston B, Clabots C, Kuskowski MA, Bender J, Smith KE, Winokur PL, Belongia EA. Antimicrobial drug–resistant *Escherichia coli* from humans and poultry products, Minnesota and Wisconsin, 2002–2004. Emerg Infect Dis. 2007;13(6):838.
33. Collignon P, Aarestrup FM, Irwin R, McEwen S. Human deaths and third-generation cephalosporin use in poultry, Europe. Emerg Infect Dis. 2013;19:1339.
34. Díez-Villaseñor C, Almendros C, García-Martínez J, Mojica FJ. Diversity of CRISPR loci in *Escherichia coli*. Microbiology. 2010;156(5):1351–61.
35. Louwen R, Staals RH, Endtz HP, van Baarlen P, van der Oost J. The role of CRISPR-Cas systems in virulence of pathogenic bacteria. Microbiol Mol Biol Rev. 2014;78(1):74–88.
36. Perez-Rodriguez R, Haitjema C, Huang Q, Nam KH, Bernardis S, Ke A, DeLisa MP. Envelope stress is a trigger of CRISPR RNA-mediated DNA silencing in *Escherichia coli*. Mol Microbiol. 2011;79(3):584–99.
37. Mohd Yusof H, Mohamad R, Zaidan UH, Abdul Rahman NA. Microbial synthesis of zinc oxide nanoparticles and their potential application as an antimicrobial agent and a feed supplement in animal industry: a review. J Anim Sci Biotechnol. 2019;10:1–22.
38. Padmavathy N, Vijayaraghavan R. Enhanced bioactivity of ZnO nanoparticles—an antimicrobial study. Sci Technol Adv Mater. 2008;9:035004.
39. Akbar A, Sadiq MB, Ali I, Muhammad N, Rehman Z, Khan MN, Muhammad J, Khan SA, Rehman FU, Anal AK. Synthesis and antimicrobial activity of zinc oxide nanoparticles against foodborne pathogens *Salmonella* Typhimurium and *Staphylococcus aureus*. Biocatal Agric Biotechnol. 2019;17:36–42.
40. Duffy LL, Osmond-McLeod MJ, Judy J, King T. Investigation into the antibacterial activity of silver, zinc oxide and copper oxide nanoparticles against poultry-relevant isolates of *Salmonella* and *Campylobacter*. Food Control. 2018;92:293–300.

Publisher's note