Original Article

First report of detection of *mcr*-1 and virulence genes in avian pathogenic *Escherichia coli* in the center of Algeria

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Abstract

Background: Antimicrobial resistance in avian pathogenic *Escherichia coli* (APEC) represents a major concern in the avian industry worldwide and limited studies have investigated Colistin resistance among APEC in Algeria. **Aims:** Investigate antibiotic resistance, in particular, Colistin, and mediated-Colistin resistance (*mcr*) genes, as well as the virulence genes in APEC. **Methods:** One hundred *E. coli* were isolated from poultry suspected of colibacillosis. Antimicrobial susceptibility testing was done on 14 antibiotics by the disk diffusion method. Colistin minimum inhibitory concentration (MIC) was assessed by the broth microdilution method. Using multiplex PCR, *mcr* genes (*mcr*-1 to 5) and 7 virulence-related genes were investigated in Colistin-resistant isolates. **Results:** Results showed high resistance to Tetracycline (99%), Nalidixic acid (92%), Doxycycline (90%), Ampicillin (89%), Ofloxacin (74%), Sulfamethoxazole-Trimethoprim (72%), and Amoxicillin-Clavulanic acid (57%); in addition, 92% of isolates were multidrug resistant. The rate of resistance to Colistin was 27% (27/100) of which 96.3% (26/27) of isolates carried the *mcr*-1 gene. Twenty-five of the Colistin-resistant isolates (92.59%) had at least three virulence genes. The most frequently isolated virulence genes were: *fimH* (96.3%) followed by *hlyF*, *iroN*, and *iss* (77.7%, each), *iutA* and *ompT* were found in 59.25% and 55.5% of isolates, respectively. The most prevalent combination of virulence factors was *hlyF-iss-iroN-iutA-ompT-fimH*. **Conclusion:** This is the first report which highlighted Colistin resistance with the detection of *mcr*-1 in APEC isolates in the area of study. Colistin resistance and carriage of *mcr*-1 in virulent and multidrug-resistant isolates of *E. coli* are alarming and a surveillance program to limit the spread of these pathogens is mandatory.

Key words: Antimicrobial resistance, Avian pathogenic *Escherichia coli*, Colistin resistance, Virulence genes

Introduction

Colibacillosis is considered a major disease affecting the chicken industry worldwide, causing high mortality and economic losses (Schouler *et al*., 2012). It often affects the respiratory tract and evolves into a systemic infection characterized by airsacculitis, pericarditis, and perihepatitis lesions (Dziva and Stevens, 2008). The infection is enhanced by stress, decreased immunity, co-infections, and poor hygiene (Dho-Moulin and Fairbrother, 1999). Colibacillosis is caused by virulent strains of *Escherichia coli*, called "Avian Pathogenic *E. coli*" (APEC) (Dho-Moulin and Fairbrother, 1999).

The pathogenicity of APEC isolates is related to the possession of several virulence factors involved in colonization, and survival of bacteria in the host (De Oliveira *et al*., 2015). In poultry, APEC has a distinct repertoire of virulence genes, which allows for the genetic diversity of these strains (Mellata, 2013; Cordoni *et al*., 2016). Among the virulence determinants, five genes located on the big virulence-plasmid ColV [*iutA* (iron transport), *hlyF* (hemolysin), *iss* (serum survival), *iroN* (siderophore iron chelation), and *ompT* (outer membrane protease)], are mostly carried by APEC, and allow to distinguish it from faecal *E. coli* in poultry (Johnson *et al*., 2008). In addition, fimbriae genes are frequently associated with APEC, facilitating the adherence of *E. coli* to host cells (Dho-Moulin and Fairbrother, 1999). Moreover, some virulence genes carried by APEC strains are related to their virulence potential in humans posing a potential threat to human health through zoonotic transmission (Ewers *et al*., 2009;

Mellata, 2013).

The abusive use of antibiotics as a fundamental strategy to treat colibacillosis led to the spread of antimicrobial resistance in APEC complicating the problem related to this pathogen (Blanco *et al*., 1997). Nowadays, this resistance is potentially transmitted between poultry flocks, and even zoonotic transfer via the food chain, representing a public health problem (Hoelzer *et al*., 2017). Thus, monitoring the emergence of bacterial drug resistance in pathogenic bacteria is necessary for optimizing effective antimicrobial treatments in poultry and humans (Dhaouadi *et al*., 2020).

Colistin is widely used to prevent and treat colibacillosis (Dandachi *et al*., 2018). It is also an important antibiotic used as a last resort for the treatment of multidrug-resistant Gram-negative bacterial infections in humans (Barbieri *et al*., 2017). For many years, resistance to Colistin was not considered a clinical problem because resistance's Colistin genetic determinants were chromosomal, and there had been no reports of horizontal transmission (Liu *et al*., 2016). The first description of the plasmid-mediated Colistin resistance (*mcr*)-1 was done in China by Liu *et al*. (2016), and there was a spread of *mcr* gene and its variants (*mcr*-1 to 10) (Wang *et al*., 2020). In addition, several reports described the implication of *mcr* genes in APEC, in Japan (Hayashi *et al*., 2019), Tunisia (Dhaouadi *et al*., 2020), European countries (Mead *et al*., 2022), and in different countries of the world (Barbieri *et al*., 2017).

In Algeria, *mcr*-1 gene was initially identified in avian faecal *E. coli* (Olaitan *et al*., 2016) and then human clinical samples (Berrazeg *et al*., 2016; Yanat *et al*., 2016). Later, other studies have reported the *mcr*-1, in different regions of Algeria from different sources: poultry (Chabou *et al*., 2016; Chabou *et al*., 2019), pigeons (Loucif *et al*., 2022), environmental samples (Touati *et al*., 2020), seawater (Drali *et al*., 2018), and fresh vegetables (Chelaghma *et al*., 2022).

In the context of raising the statement of emergence and dissemination of antimicrobial resistance in Algeria, and contributing to updating the current data on the genetic determinants among virulence and plasmid-mediated Colistin resistance in APEC strains, the present study was conducted aiming to (1) determine the frequency of antimicrobial resistance in *E. coli* isolated from poultry affected by colibacillosis in the center of Algeria, (2) investigate their phenotypic and genotypic Colistin resistance, and (3) characterize the virulence-associated factors among Colistin-resistant isolates.

Materials and Methods

Samples collection

From October 2019 to October 2021, 164 samples were gathered from poultry suspected of colibacillosis disease (broilers (105), layers (39), and broiler and layer breeders (20)). Poultry farms were located in four provinces in the center of Algeria (Chlef, Ain Defla, Blida, and Algiers). After the necropsy, organs (liver, spleen, heart, and lungs) were removed and quickly delivered to the laboratory, on ice at 4°C, for the microbiological survey.

E. coli isolation and characterization

Small pieces of the different organs were placed in brain heart infusion (BHI) broth. After an incubation of 18 h at 37°C, a drop was inoculated on Hektoen agar, and these plates were incubated at 37°C for 24 h. The characteristic salmon yellow colonies obtained were purified on nutrient agar. The API 20E system® (BioMérieux, France), was then used to identify the species of *E. coli*. The purified colonies of *E. coli* were transferred into BHI broth with 30% glycerol and then kept at -20°C till further usage.

Antimicrobial susceptibility test and detection of extended-spectrum β-lactamases (ESBL) production

To conduct testing for antimicrobial susceptibility, the disk diffusion method was used in compliance with the Clinical and Laboratory Standards Institute (CLSI) guideline (CLSI, 2018). For this purpose, 14 antibiotics were tested: Ampicillin (AMP) 10 µg, Amoxicillin-clavulanic acid (AMC) 20/10 µg, Cefoxitin (FOX) 30 µg, Cefotaxime (CTX) 30 µg, Aztreonam (ATM) 30 µg, Ceftazidime (CAZ) 30 µg, Tetracycline (TET) 30 µg, Doxycycline (DOX) 30 µg, Nalidixic acid (NAL) 30 µg, Ofloxacin (OFL) 5 µg, Trimethoprim-Sulfamethoxazole (SXT) 1.25/23.75 µg, Gentamicin (GEN) 10 µg, Nitrofurantoin (F) 300 µg, and Chloramphenicol (CHL) 30 µg. The double disk synergy method was used to screen the ESBL production (CLSI, 2018). The isolates were classified as multidrug-resistant when a lack of susceptibility to at least one drug across three or more antimicrobial classes was observed (Magiorakos *et al*., 2012).

Determination of Colistin resistance

The *E. coli* isolates (n=100) were screened for minimum inhibitory concentration (MIC) using the broth microdilution assay (BMD) according to the CLSI guidelines (CLSI, 2012). Successive two-fold dilutions of Colistin ranged from 16 to 0.03 µg/ml. Resistance to Colistin was considered when a MIC >2 µg/ml was found (EUCAST, 2022). For quality control, the *E. coli* ATCC 25922 strain was used.

DNA extraction

The DNA extraction was carried out using the boiling procedure. Briefly, 2-3 fresh colonies were suspended in 150 µL of nuclease-free water. The supernatant containing DNA was obtained after 10 min of heating at 98°C, followed by centrifugation at 12000 g, according to a previous description by Dilhari *et al*. (2017). DNA purity was checked by a spectrophotometer (NanoDrop, 8000, Thermo Scientific) and DNA was kept at -20°C until use.

Detection of *mcr* genes and virulence factors associated with genes

Multiplex polymerase chain reaction (PCR) was used to detect the *mcr* genes (1 to 5), in Colistin resistant isolates. It was conducted at the Biotechnology Research Center of Constantine, Algeria (CRBt). Table 1 lists the PCR primers utilized. The PCR reaction was performed according to Rebelo *et al*. (2018).

Seven APEC virulence factors associated with genes were investigated by simplex PCR among Colistin-resistant *E. coli*. The primer sequences of target genes are summarized in Table 2.

The *iss*, *ompT*, *iroN*, and *hlyF* genes were detected using the PCR protocol described before by Johnson *et al*. (2006). The cycling conditions of the *iutA* gene were performed as described previously by Johnson and Stell (2000), *fimH* and *papC* genes were detected using the protocol described by Al-Kandari and Woodward (2019).

Statistical analysis

Descriptive analysis and statistical tests were done by SPSS, IBM ® software; V.25 2015. The correlation of resistance to antibiotics and multi-drug resistance profile, with APEC profile, was analysed using Pearson's Chi-squared test (X2), and Fisher's exact test (when expected values are <5).

Results

Antimicrobial susceptibility test

164 samples of colibacillosis cases, yielded a collection of 100 *E. coli* isolates, giving a percentage of 60.97%. These isolates originated from 77 broilers, 17 layers, and 6 broiler and layer breeders.

For the total number of isolates, the results of antimicrobial resistance showed high resistance to Tetracycline (99%), Nalidixic acid (92%), Doxycycline (90%), Ampicillin (89%), Ofloxacin (74%), Trimethoprim-Sulfamethoxazole (72%), and Amoxicillin-Clavulanic acid (57%), as shown in Fig. 1. Moreover, no ESBL resistance was detected in any of the isolates. Interestingly, 92% of isolates showed a multidrug resistance profile, and 31 of those showed resistance to more than 6 antibiotic classes.

Colistin MIC and *mcr* genes detection

Among the 100 *E. coli* isolates, 27 were Colistin-resistant (MIC >2 µg/ml), and they revealed MICs of 4 µg/ml (6 isolates), 8 µg/ml (12 isolates), 16 µg/ml (7 isolates), and 2 isolates with MIC >16 µg/ml. It should be pointed out that the antimicrobial resistance

Table 1: Characteristics of *mcr*-genes primers used in the study

Gene	Primer sequences	Size (bp)	Reference
mcr-1	F: AGTCCGTTTGTTCTTGTGGC R: AGATCCTTGGTCTCGGCTTG	320	Rebelo *et al*. (2018)
mcr-2	F: CAAGTGTGTTGGTCGCAGTT R: TCTAGCCCGACAAGCATACC	715	
mcr-3	F: AAATAAAAATTGTTCCGCTTATG R: AATGGAGATCCCCGTTTTT	929	
mcr-4	F: TCACTTTCATCACTGCGTTG R: TTGGTCCATGACTACCAATG	1116	
mcr-5	F: ATGCGGTTGTCTGCATTTATC R: TCATTGTGGTTGTCCTTTTCTG	1664	

Table 2: Characteristics of primers used in virulence genes investigation

Gene	Primer sequences	Size (bp)	Reference
papC	F: TGATATCACGCAGTCAGTAGC R: CCGGCCATATTCACATAA	501	Al-Kandari and Woodward (2019)
ompT	F: ATCTAGCCGAAGAAGGAGGC R: CCCGGGTCATAGTGTTCATC	559	Johnson *et al*. (2006)
iroN	F: AAGTCAAAGCAGGGGTTGCCCG R: GACGCCGACATTAAGACGCAG	667	Johnson *et al*. (2006)
hlyF	F: GGCGATTTAGGCATTCCGATACTC R: ACGGGGTCGCTAGTTAAGGAG	599	Johnson *et al*. (2006)
fimH	F: AGAACGGATAAGCCGTG R: GCAGTCACCTGCCCTCCGGTA	508	Al-Kandari and Woodward (2019)
Iss	F: CAGCAACCCGAACCACTTGATG R: AGCATTGCCAGAGCGGCAGAA	323	Johnson *et al*. (2006)
iutA	F: GGCTGGACATCATGGGAACTGG R: CGTCGGGAACGGGTAGAATCG	302	Johnson and Stell (2000)

Table 3: Virulence genes and resistance phenotype combinations in Colistin-resistant isolates

Virulence genes	Number of virulence factors	Resistance phenotypes	Number of antibiotics	Sample's origin	Number of isolates
hlyF-iss-iroN-iutA-ompT-fimH	6	AMP-NAL-OFL-SXT-TET-DOX-CHL-COL	8	Broiler	1
				Layer	1
		AMP-NAL-OFL-TET-DOX-COL	6	Broiler	1
		AMP-AMC-NAL-OFL-SXT-TET-DOX-COL	8	Broiler	2
hlyF-iss-ompT-iroN-fimH-papC	6	AMP-NAL-OFL-SXT-TET-DOX-CHL-COL	8	Broiler-breeder	1
hlyF-iss-iroN-iutA-fimH	5	AMP-AMC-NAL-OFL-SXT-TET-DOX-COL	8	Broiler	1
		NAL-SXT-TET-DOX-COL	5	Broiler	1
		AMP-AMC-NAL-TET-DOX-COL	6	Broiler	1
hlyF-iss-ompT-iroN-fimH	5	AMP-AMC-NAL-OFL-SXT-TET-DOX-COL	8	Broiler	2
		AMP-AMC-NAL-OFL-SXT-TET-COL	7	Broiler	1
hlyF-iss-ompT-iutA-fimH	5	AMP-NAL-OFL-SXT-TET-DOX-CHL-COL	8	Broiler	1
HlyF-ompT-iutA-fimH	4	AMP-NAL-OFL-SXT-TET-DOX-F-CHL-COL	9	Broiler	1
		AMP-NAL-OFL-SXT-TET-DOX-CHL-COL	8	Broiler	2
hlyF-iss-iroN-fimH	4	AMP-AMC-NAL-OFL-SXT-TET-DOX- CHL-COL	9	Broiler	3
		AMP-AMC-NAL-OFL-SXT-TET-DOX-COL	8	Broiler	1
hlyF-iroN-iutA-fimH	4	AMP-NAL-OFL-SXT-TET-DOX-COL	7	Broiler	1
Iss-iroN-iutA-fimH	4	AMP-AMC-NAL-OFL-SXT-TET-DOX-COL	8	Broiler	2
				Layer	1
Iss-ompT-iroN	3	AMP-NAL-TET-DOX-COL	5	Broiler	1
ompT-fimH	2	AMP-AMC-SXT-TET-DOX-COL	6	Layer	1
fimH	1	AMP-NAL-OFL-SXT-TET-DOX-COL	7	Broiler	1
Total number of Colistin-resistant isolates					27

CHL: Chloramphenicol, AMP: Ampicillin, AMC: Amoxicillin-Clavulanic acid, OFL: Ofloxacin, SXT: Sulfamethoxazole-Trimethoprim, NAL: Nalidixic acid, TET: Tetracycline, DOX: Doxycycline, F: Nitrofurantoin, and COL: Colistin

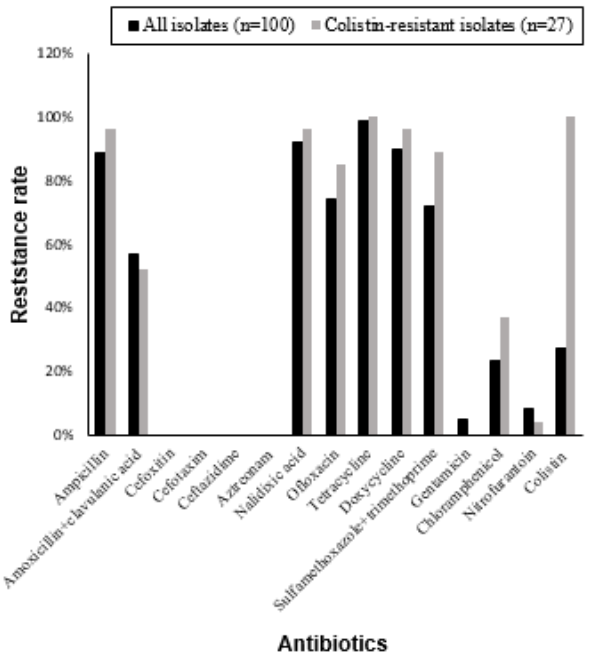

Fig. 1: Results of antimicrobial susceptibility testing

profile of Colistin-resistant isolates has the same tendency as observed for all isolates, except for Gentamicin, where no resistance was found, as shown in Fig. 1.

Therefore, 11 different profiles of antimicrobial resistance were observed in Colistin-resistant isolates, the most prevalent profiles were: AMP-NAL-OFL-SXT-TET-DOX-CHL-COL (6 isolates) and AMP-AMC-OFL-SXT-NAL-TET-DOX-COL (8 isolates). Most interestingly, all Colistin-resistant isolates were multidrug-resistant, as shown in Table 3.

Among the 27 Colistin-resistant isolates, 26 (96.29%) harboured the *mcr*-1 gene without the detection of the others (2 to 5). One isolate with a MIC >16 µg/ml tested negative for *mcr*-1, this high level of resistance suggests that the mechanism underlying is chromosome-mediated Colistin resistance. The isolates with the *mcr*-1 gene were from broilers (22 isolates), layers (3 isolates), and broiler breeders (1 isolate).

Virulence factors associated with genes

Among the 27 Colistin-resistant isolates found in this study, 25 (92.59%) had at least 3 virulence factors genes. The virulence factor genes: *hlyF*, *iroN*, and *iss* were equally detected in 21 isolates, followed by *iutA* in 16 isolates and *ompT* in 15 isolates. In addition, *fimH* was highly detected, in 26 isolates. However, *papC* was found in only one isolate. The virulence gene combinations detected are shown in Table 3, the predominant combinations observed in APEC isolates were *hlyF-iss-iroN-iutA-ompT-fimH* (5 isolates), and *hlyF-iss-ompT-iroN-fimH* (4 isolates).

The most prevalent profile associating virulence factors and antibiotic resistance in *mcr*-1 resistant *E.*

coli is *hlyF-iss-iroN-fimH*/AMP-AMC-NAL-OFL-SXT-TET-DOX-CHL-COL found in three isolates.

Statistical analyses revealed no correlation between antibiotic resistance, multi-drug resistance profiles, and the APEC profile of the isolates (P>0.05).

Discussion

In this study, we were interested in detecting Colistin-resistant isolates and associated virulence factor genes among APEC isolated in poultry farms in the center regions of Algeria. Indeed, previous investigations carried out in the area of the study were mainly focused on the detection of *mcr* genes in avian faecal *E. coli* isolates (Chabou *et al*., 2016, 2019); thus, the present study is the first for the simultaneous detection of *mcr* and virulence factor genes in APEC in the center of Algeria.

The profile of multidrug resistance of *E. coli* isolated from poultry samples and the important rates of resistance to the tested antimicrobial agents, agree with previous works carried out in Algeria (Meguenni *et al*., 2015) (100%), (Halfaoui *et al*., 2017) (98.7%), (Mohamed *et al*., 2018) (100%), and (Meguenni *et al*., 2019) (53.5%), with high levels of resistance to Tetracycline, Ampicillin, Ofloxacin, and Sulfamethoxazole-Trimethoprim. With the same tendency, 90.4% and 96% of the isolates were multidrug resistant, respectively in Egypt (Radwan *et al*., 2014) and Tunisia (Dhaouadi *et al*., 2020). In Algeria, antibiotics are frequently used to prevent and treat colibacillosis (Dandachi *et al*., 2018; Mohamed *et al*., 2018). Consequently, the abuse of antibiotics in poultry farms may contribute to the acquisition of resistance to different molecules, especially when any antimicrobial susceptibility testing is not applied (Varga *et al*., 2018). Tetracycline and Fluoroquinolones are incontestably antibiotics with high levels of resistance, due to their availability and broad spectrum, these antibiotics are widely used in veterinary medicine in Algeria (Mohamed *et al*., 2018).

Our findings showed a high prevalence of Colistin resistance (27%), which was also reported in North African countries such as Tunisia (24%) (Dhaouadi *et al*., 2020), and Egypt (25%) (Badr *et al*., 2022). However, a recent report carried out by Mead *et al*. (2022) in several European countries showed a low proportion of phenotypic resistance to Colistin in *E. coli* isolated from poultry farms. The difference in resistance to Colistin between countries may be related to the different husbandry practices applied in European and North African countries (Barbieri *et al*., 2017).

This study revealed that the *mcr*-1 gene was the main Colistin resistance determinant detected. Several reports indicated that Colistin resistance is primarily mediated by the *mcr*-1 gene, the results of Chaalal *et al*. (2021) showed that the *mcr*-1 gene was detected in all Colistin-resistant *E. coli* isolates. In Tunisia, 7 out of 12 isolates with Colistin resistance carried the *mcr*-1 gene (Dhaouadi *et al*., 2020). In a systematic review that included studies carried out in North Africa, among a total of 208 *mcr*-positive strains, 193 (92.8%) have the *mcr*-1 gene (Touati and Mairi, 2021).

The resistant isolates in this investigation had MIC values that ranged between 4 and ≥16 µg/ml. Such findings are in agreement with those of other studies that reported the same MICs (Azam *et al*., 2017; Amin *et al*., 2020; Dhaouadi *et al*., 2020). It is known that *mcr*-1 gene is generally associated with low MICs values comprising from 2 to 8 µg/ml (Liu *et al*., 2016). Therefore, in our study *mcr*-1 was found in one isolate from two isolates with MIC >16 µg/ml; it was suggested that mutations in sensor kinases related to Colistin-resistance pmrB and phoQ may also occur in isolates that are *mcr*-1 positive and modulate the MIC to Colistin (Kim *et al*., 2019; Al-Mir *et al*., 2021; Zhu *et al*., 2021).

Additionally, our investigation revealed that a significant number of isolates harbored virulence determinants that involved iron acquisition, adhesion, serum resistance, and expression of toxins. The most frequently found genes were *iroN*, *iss*, and *hlyF* (77.77%). These were in agreement with the previous studies in Algeria (Mohamed *et al*., 2018; Meguenni *et al*., 2019). In Egypt, similar results were found for *iroN*, *iss* genes (80.02% each) (Ahmed *et al*., 2013) and *iroN*, *hlyF* genes (80% each) (Radwan *et al*., 2014).

The *iutA* and *ompT* were detected at rates of 59.25% and 55.55%, respectively. The results differ from one study to another, the *iutA* was highly detected by Ahmed *et al*. (2013) (78.0%), contrarily to Radwan *et al*. (2014) (5%), and Meguenni *et al*. (2019) (13.9%). The *ompT* detection rate was in accordance with the study of Meguenni *et al*. (2019) but lower than reported by Mohamed *et al*. (2018). The occurrence of virulence genes may differ between pathotypes and regions (Maturana *et al*., 2011).

Based on the combination of the five virulence genes claimed by Johnson *et al*. (2008) as markers of APEC, more than 90% of our isolates harbored at least three virulence factors associated genes. These findings were in accordance with previous results reported in Algeria (Mohamed *et al*., 2018; Meguenni *et al*., 2019), and other countries (Ahmed *et al*., 2013; Radwan *et al*., 2014; Varga *et al*., 2018).

Regarding the fimbriae genes, more than 96% of our isolates had the *fimH* gene. This agreed with the results of Delicato *et al*. (2003), Al-Kandari and Woodward (2019), and Xu *et al*. (2019). The FimH adhesin enables colonization and the development of biofilms (Sarowska *et al*., 2019), and it enhances the ability of APEC bacteria to adhere and colonize host cells in the first few days of infection (Pourbakhsh *et al*., 1997; Dho-Moulin and Fairbrother, 1999).

However, this does not claim that all strains having *fimH* are potentially virulent (Al-Kandari and Woodward, 2019).

APEC strains can be genetically very diverse and have a unique repertoire of virulence genes (Mellata, 2013; Cordoni *et al*., 2016). Isolates studied are certainly diverse with different pathogenicity patterns but some of them are considered as potentially highly virulent since they possess four or five virulence genes (Johnson *et al*., 2008; Vounba *et al*., 2018).

Our study revealed that Colistin-resistant *E. coli* having *mcr*-1 gene is largely disseminated in the poultry farms located in the center of Algeria. These isolates are resistant to diverse antibiotic classes and present a serious virulence potential. This represents a public health problem since Colistin is a critical antibiotic used in human medicine. As far as we can tell, this study is the first to describe the concomitant presence of *mcr*-1 and the virulence factor genes in APEC isolated from colibacillosis cases in poultry farms in Algeria. The establishment of a surveillance program to trace the emergence of virulent and antibiotic-resistant *E. coli* strains in poultry farming is mandatory. Additionally, efforts are needed to monitor the spread of bacteria among animals and their environment to avoid potential zoonotic transmission to humans.

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Conflict of interest

The authors declare that they have no conflict of interest.

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