



Fluoroquinolone-resistant *Escherichia coli* strains in animal and poultry feed from Ukrainian feed-producing enterprises

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The article presents the results of studying the sensitivity of *Escherichia coli* strains isolated from animal and poultry feed samples to fluoroquinolones of the second (ofloxacin, norfloxacin), third (levofloxacin) and fourth (moxifloxacin) generations. It was shown that high resistance to norfloxacin was detected in 7 strains of isolated *E. coli*, which accounted for 33.3 % of the identified ones. To ofloxacin and to the representative of the third generation of fluoroquinolones — levofloxacin, 3 isolated strains of *E. coli* were resistant, which was 14.3 % of the studied strains. Resistance to the representative of the fourth generation of fluoroquinolones — moxifloxacin — was detected in 1 strain of *E. coli*, which was 4.8 % of the tested strains. The results of the studies on fluoroquinolone resistance of the isolated *E. coli* strains indicate a significant contamination of animal and poultry feed with fluoroquinolone-resistant escherichia. This poses potential risks for the spread and possible transmission of such resistance to other bacterial species and the normobiota of the gastrointestinal tract of animals, poultry when ingested with feed and humans when consuming products of animal origin after feeding such feed.

Key words: *Escherichia coli*, fluoroquinolones, mixed fodder, bran, meal, premixes, animal meal, fish meal

Introduction

Achieving the welfare of animals and poultry, ensuring their high productivity, their resistance to bacterial diseases, high livestock safety, and obtaining safe animal products is impossible without the use of high-quality, safe and nutritious feed [16, 19]. Scientists emphasize the need to create a strong feed base, which includes a system and structure for the production of biologically safe feed and is one of the main conditions for ensuring food security in Ukraine [9, 12]. However, a number of researchers emphasize the problem of contamination of feed with bacterial pathogens, including zoonotic pathogens, during their production, at critical points of certain technological

processes, due to the lack of prediction of contamination of individual components of feed used as raw materials from other agricultural sectors [7, 14]. Although Ukraine has a system for monitoring raw materials and products of the entire agricultural sector, monitoring of animal and poultry feed does not cover all possible risks. The resistance of pathogenic microorganisms to antibiotics isolated from cattle, pig and poultry feed, feed materials, dry and canned feed remains an extremely urgent problem today. This is due to the danger of antibiotic resistance transmission to other species of animals, poultry, and humans [2, 4]. In the last decade, bacterial microorganisms have developed resistance mechanisms that have seriously limited the effectiveness of conventional antibiotic therapy [3].

It should be recalled that Ukraine has implemented the National Action Plan to Combat Antimicrobial Resistance in accordance with the provisions of the Global Strategy of the World Health Organisation (WHO) to curb antibiotic resistance. However, the Plan does not cover Ukrainian feed production enterprises.

Scientists point out that *Escherichia coli* is included in the list of pathogens that the WHO considers to be resistant pathogens [6]. In particular, *E. coli* is able to develop resistance to clinically important antibiotics — fluoroquinolones. The mechanism of action of fluoroquinolones is based on the specific inhibition of DNA gyrase (for gram-negative microorganisms) and topoisomerase IV for gram-positive bacteria [3].

The aim of our work was to test *E. coli* strains isolated from animal and poultry feed samples for susceptibility to clinically important representatives of fluoroquinolones of the II (ofloxacin, norfloxacin), III (levofloxacin) and IV (moxifloxacin) generations and to determine the prevalence of fluoroquinolone-resistant escherichia.

Materials and Methods

The study was carried out in the Scientific Research Microbiology Department of SSRILDVSE (Kyiv) and the Institute of Animal Biology NAAS (Lviv).

As a result of microbiological studies of 382 samples of various types of animal and poultry feed, 21 *E. coli* strains were isolated and identified. In particular, among 36 samples of premixes, *E. coli* was isolated in 2 cases; among 47 samples of mixed fodder and bran — in 7 cases; among 127 samples of meal and cake — in 6 cases; among 88 samples of other feeds — in 6 cases, *E. coli* was isolated from grain samples [7].

In the studies to determine the susceptibility of *E. coli* of the isolated strains to fluoroquinolones, discs with a certain antibiotic concentration were used according to the latest EUCAST recommendations, in particular, ofloxacin (5 µg) and norfloxacin (10 µg) — second-generation fluoroquinolones, levofloxacin (5 µg) — third-generation fluoroquinolones and moxifloxacin (5 µg) — fourth-generation fluoroquinolones [17].

All antibiotic disks are manufactured by *Himedia Laboratories Pvt. Limited* (India) with the appropriate expiration dates. Antibiotic disks are registered in Ukraine and meet international quality standards ISO, CE, WHO GMP [17].

In accordance with the recommendations of the EUCAST version, before setting up experiments to determine the susceptibility to fluoroquinolones of the isolated *E. coli* strains, regular and extended internal quality control was performed to determine the diffusion of disks with fluoroquinolones at their respective concentrations: ofloxacin (5 µg), norfloxacin (10 µg), levofloxacin (5 µg), moxifloxacin (5 µg) [18].

Mueller Hinton Agar M173 (Mueller-Hinton agar) with a pH in the range of 7.2–7.4 was used for testing according to EUCAST recommendations. The manufacturer is *Himedia*,

the batch used has been tested and standardized in accordance with the requirements of CISI-M6: Protocol for the evaluation of dry Mueller Hinton agar. The composition of the Mueller-Hinton agar on a g/l basis includes: meat infusion 300.0; acid hydrolyzate of casein 17.5; starch 1.50; agar-agar 17.00. The agar was prepared according to the described method: 38.0 g of powder was dissolved in 1000 ml of distilled water, boiled until the powder was completely dissolved; sterilized at 1.1 atm (121°C) for 15 min. After sterilization, the Mueller-Hinton agar was thoroughly mixed and poured into Petri dishes with an agar column height of 4.0±0.5 mm (about 25.0 cm³). After complete solidification of the agar, the dishes were dried to remove condensation. Ready-made Mueller-Hinton agar plates were checked for sterility and suitability for *E. coli* growth. To control the sterility, the plates with Mueller-Hinton medium were left in a thermostat at 37.0±1.0°C for 24 hours. Control of the growth properties of Mueller-Hinton medium was checked by using a test culture of *E. coli* ATCC 25922 for inoculation, followed by cultivation in a thermostat at 37.0±1.0°C for 24 hours and determination of the growth rate of the culture.

For regular extended internal control of the determination of diffusion of fluoroquinolone disks, the test culture of *E. coli* ATSS 25922, recommended by EUCAST for microorganisms of the *Enterobacterales* order, was used. The test culture of *E. coli* was inoculated on MPA and after 24 h of incubation in a thermostat at 37±0.5°C, the agar of the grown daily culture was washed off with sterile saline under aseptic conditions. Using a turbidity detector for bacteriological suspensions (Densi-La-Meter *Lachema*), the inoculums were brought to a concentration of 0.5 McFarland optical units with sterile saline.

The results of the routine and extended internal quality control for determining the diffusion of fluoroquinolone disks were recorded by measuring the diameter of the zones of growth inhibition of the test culture of *E. coli* ATCC 25922 and checking their compliance with the values recommended by EUCAST [18].

To determine the susceptibility to the indicated fluoroquinolones, inoculums of the isolated *E. coli* strains were prepared in a similar way (as well as the test culture).

The prepared bacterial inocula of the isolated *E. coli* strains were applied to bacterial plates with Mueller-Hinton agar in a volume of 0.1 cm³ per plate. Inoculation of the respective bacterial suspensions into the agar was performed using a sterile swab, which was previously moistened in the respective inoculum. The applied inoculum of escherichia was thoroughly rubbed into the agar surface. Rubbing was performed by rotating the test dish in a circle. The inoculated bacterial dishes were kept at room temperature for about 15 minutes to diffuse the respective bacterial inocula into the agar. The antibiotic disks were applied to the surface of the inoculated bacterial dishes (4 for each dish). Incubation of the cups with cultures and antibiotic disks was carried out for 20 h in a thermostat at a temperature of 35±1°C.

The results were recorded by measuring the diameter of the growth inhibition zones of each *E. coli* strain around

the disk with the corresponding antibiotic. The zone of growth inhibition of *Escherichia* strains was clear, without any growth within it. To measure the growth inhibition zones, the bacterial dish with the lid closed was placed upside down over a dark matte surface at an angle of 45°, creating a reflected light effect. The growth inhibition zone was measured using a caliper to the nearest millimeter.

The results were interpreted in accordance with the current version of EUCAST according to the Breakpoint Tables for the interpretation of the diameters of the zones of growth inhibition of cultures [17].

Research Results

The results of routine quality control of disks containing fluoroquinolones ofloxacin, norfloxacin, levofloxacin, and moxifloxacin are presented in table 1.

The results of the quality control of the diffusion of disks with ofloxacin, norfloxacin, levofloxacin and moxifloxacin showed that the diameters of the growth inhibition zones under their action on the test culture of *E. coli* ATCC 25922 were within the permissible values. In particular, according to EUCAST, the range of permissible values of the size of the zone of growth inhibition of *E. coli* ATCC 25922 for ofloxacin is within 29–33 mm. During the quality control of the disks with ofloxacin, the diameter of the growth inhibition zone was 32 mm, which confirmed the compliance of the drug with EUCAST requirements. According to the results of the quality control of the diffusion of disks with norfloxacin, its compliance with EUCAST requirements was confirmed, since the diameter of the growth inhibition zones of the test culture of *E. coli* was 31 mm, with the permissible range of values from 28 to 35 mm. The quality of diffusion of moxifloxacin disks was confirmed by the diameter of the zone of growth inhibition of the test culture of *E. coli* at 30 mm with a range of permissible values from 28 to 35 mm. Based on the analysis of the results of the control of diffusion of disks with fluoroquinolones, these antibacterial drugs were

approved for use in further studies of the sensitivity of the isolated *E. coli* strains.

According to the analysis of the results of studies on the sensitivity of 21 (twenty-one) identified *E. coli* strains to fluoroquinolones, the highest resistance to norfloxacin was found in 7 (33.3 %) strains of *E. coli*. It should be noted that the above-mentioned strains of *E. coli* were isolated from samples of feed bran (2 strains), wheat grain (2), sunflower sprats (2) and protein-mineral-vitamin supplements for poultry (1 strain). Importantly, the *E. coli* strain Ec18 isolated from wheat bran was completely insensitive to norfloxacin, as evidenced by the continuous growth of *Escherichia* colonies around the antibiotic disk.

It should also be noted that in *E. coli* strain Ec49, isolated from sunflower meal, developed resistance to norfloxacin in individual bacteria among the susceptible population. This was confirmed by the growth of single colonies in the zone of crop growth inhibition, the diameter of which was 33 mm, and the zone without colony growth was 24 mm (table 2).

It should be noted that among the isolated *Escherichia*, strain Ec18 isolated from wheat bran was found to be completely resistant to norfloxacin, a representative of the second-generation fluoroquinolones (fig. 1).

The results of tests to study the sensitivity of isolated *E. coli* to ofloxacin showed the resistance of 3 (14.3 % of the identified) strains of *E. coli* Ec31, Ec42, and Ec49 isolated from samples of wheat bran and sunflower meal.

According to the results of the study, 33.3 % of *E. coli* strains isolated from animal and poultry feed samples had a high level of resistance to norfloxacin and 3 strains of *E. coli* showed resistance to ofloxacin. At the same time, resistance to ofloxacin continued to develop among susceptible bacteria in the *E. coli* population. This was confirmed by the growth of single colonies in the zone of growth inhibition. The strain Ec49 was re-identified, in which, under the action of ofloxacin, bacteria with resistance to ofloxacin were formed in the sensitive population of *E. coli*. This was confirmed by the results of the study, as the diameter of the zone of inhibition of culture growth

Table 1. Results of regular extended internal quality control for the determination of diffusion of fluoroquinolone disks with *E. coli* test culture ATCC 25922 according to EUCAST

Antibiotic	According to EUCAST recommendations (Version 13.2, 2023)		Results of studies of regular extended internal quality control of diffusion of disks with fluoroquinolones:		
	Concentration of the drug, µg	Diameter of the inhibition zone growth of the test culture, mm	with the test culture of <i>E. coli</i> ATCC 25922 (according to the latest EUCAST recommendations for the study of the sensitivity of bacteria of the genus <i>Enterobacterales</i> to fluoroquinolones)		
		range of permissible values, mm	Diameter of growth inhibition zones, mm	conformity recommendations EUCAST	Conclusions
Ofloxacin	5	29–33	32	is within the range of permissible values	admission to the main experiment
Norfloxacin	10	28–35	31	— "" —	— "" —
Levofloxacin	5	29–37	33	— "" —	— "" —
Moxifloxacin	5	28–35	30	— "" —	— "" —

Table 2. Results of tests for fluoroquinolone susceptibility of *E. coli* strains isolated from animal and poultry feed samples from feed production enterprises in Ukraine, mm (n strains = 21)

No.	Type of feed from which isolated and identified strains of <i>E. coli</i>	Isolated strains of <i>E. coli</i>	Fluoroquinolones used in the experiment:							
			Ofloxacin (5 µg) II generation		Norfloxacin (10 µg) II generation		Levofoxacin (5 µg) III generation		Moxifloxacin (5 µg) IV generation	
			Diameter inhibition. strain growth, mm	interpretation	Diameter inhibition. strain growth, mm	interpretation	Diameter inhibition. strain growth, mm	interpretation	Diameter inhibition. strain growth, mm	interpretation
According to EUCAST requirements										
Range of permissible values of the diameter of the disk diffusion zone according to the EUCAST checkpoint table, mm			S≥ R< 24 22		S≥ R< 24 24		S≥ R< 25 22		S≥ R< 22 22	
Research results										
1	Protein, mineral and vitamin supplement for poultry	Ec15	31	S	20	R	28	S	20	R
2	Compound feed for cattle	Ec16	30	S	28	S	25	S	23	S
3	Wheat bran	Ec18	22	S	*c.rg.	R	20	R	25	S
4	Pre-starter food «Piglet»	Ec21	30	S	26	S	18	R	31	S
5	Wheat fodder bran	Ec27	27	S	31	S	28	S	29	S
6	Sunflower meal	Ec31	15	R	30	S	23	S	25	S
7	Wheat fodder bran	Ec42	15	R	30	S	26	S	25	S
8	Sunflower meal	Ec49	**21/ (30)	R	**24/ (33)	R	26	S	27	S
9	Vitamin and mineral premix for calves TC VMP TSB 1 %	Ec53	23	S	28	S	28	S	25	S
10	Corn grain	Ec55	23	S	28	S	28	S	25	S
11	Sunflower meal	Ec57	27	S	28	S	25	S	24	S
12	Wheat fodder bran	Ec58	26	S	30	S	21	R	30	S
13	Soybean meal	Ec59	25	S	31	S	25	S	29	S
14	Sunflower meal	Ec61	28	S	28	S	24	S	32	S
15	Corn grain	Ec64	26	S	28	S	25	S	24	S
16	Corn grain	Ec65	29	S	33	S	28	S	28	S
17	Wheat fodder bran	Ec66	28	S	22	R	30	S	24	S
18	Corn grain	Ec67	30	S	28	S	26	S	30	S
19	Wheat grain	Ec68	26	S	20	R	24	S	27	S
20	Sunflower meal	Ec69	26	S	22	R	27	S	24	S
21	Wheat of 2 and 3 classes	Ec70	26	S	19	R	26	S	22	S
ALL fluoroquinolone-resistant strains:			3		7		3		1	

Note. S — sensitive; R — resistant *c.gr. — continuous growth of bacteria to the disk (no growth inhibition zone); **21/(30) — a clear zone of growth inhibition of 21 mm with the formation of a growth inhibition zone of 30 mm (single colonies of *E. coli* grew from 30 mm to 21 mm)

of this strain was 30 mm, and decreased to 21 mm due to the growth of single culture colonies in the zone of inhibition. In *E. coli* strains Ec31, Ec42, the diameter of the growth inhibition zone was limited and amounted to 15 mm, while the range of permissible values according to EUCAST was less than 22 mm (fig. 2).

The analysis of the test results showed that the level of resistance of 3 (14.3 % of the identified) strains of *E. coli* Ec18, Ec21 and Ec58 was quite high to the representative of the third generation of fluoroquinolones — levofloxacin.

Resistance to moxifloxacin was detected in one strain of *E. coli* Ec15 (4.8 % of the identified strains). The risk of spreading *E. coli* resistant to IV generation fluoroquinolones, as recognized antibiotics with high bactericidal activity, is enhanced by the fact that this strain was isolated from samples of protein-mineral-vitamin supplement for poultry and is likely to contaminate a large percentage of poultry, transmit its resistance to other populations of bacterial microorganisms and therefore be dangerous to human health, other animal and poultry species.

Discussion

A number of researchers still hold the opinion of the high bactericidal efficacy of third-generation fluoroquinolones and are convinced that they have high bactericidal activity against gram-negative and gram-positive microorganisms; bacterial pathogens sensitive to penicillin; penicillin-resistant *Streptococcus pneumonia* and show bactericidal activity against atypical pathogens. The results of our research and that of other scientists show that sensitivity is decreasing and resistance is being developed in microorganisms to all generations of fluoroquinolones.

Scientists report that in the study of animal feed and mixed fodder, the species composition of the microflora is represented by *Enterobacter* spp., *Klebsiella* spp., *E. coli*, *Pseudomonas aeruginosae*. The largest share was made up of *E. coli* isolates. According to the authors, during the period 2019–2021, there was a tendency to increase the number of *E. coli* resistant to antibacterial drugs, the share of which in 2021 amounted to 26.6 % of the studied feed samples. Scientists emphasize that among *E. coli* isolates, the activity of resistance to fourth-generation fluoroquinolones is increasing, which until now have been characterized by high bactericidal activity against gram-positive, gram-negative, anaerobic, acid-fast microorganisms, as well as atypical pathogens, peptonococci, peptinostreptococci [8].

Resistance to moxifloxacin was detected according to the results of our studies in the case when *E. coli* was isolated and identified in a sample of poultry feed.

Scientists emphasize that fluoroquinolones have a rapid and effective bactericidal effect, have good penetration into organs and tissues, and ensure a high level of drug concentration in the blood. Currently, fluoroquinolones are considered to be one of the most bactericidally effective drugs to which bacterial pathogens are very sensitive and only in a few cases have they developed resistance to

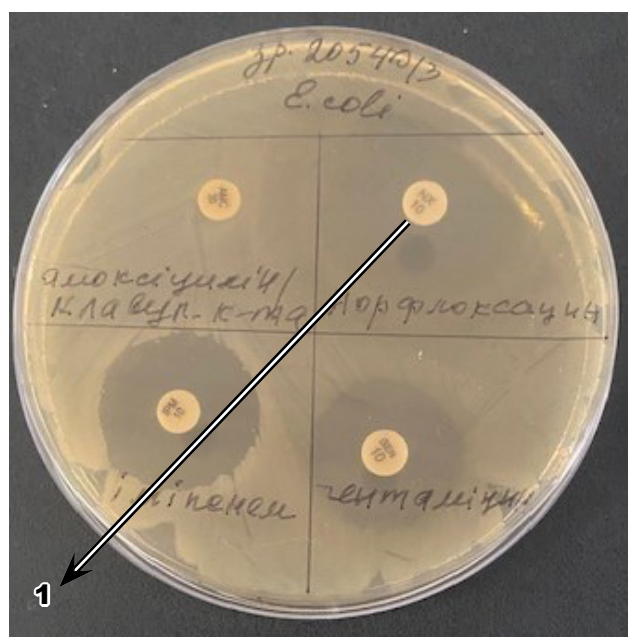


Fig. 1. Norfloxacin resistance of *E. coli* strain Ec18 isolated from wheat bran

Note. 1 — no zone of growth inhibition.

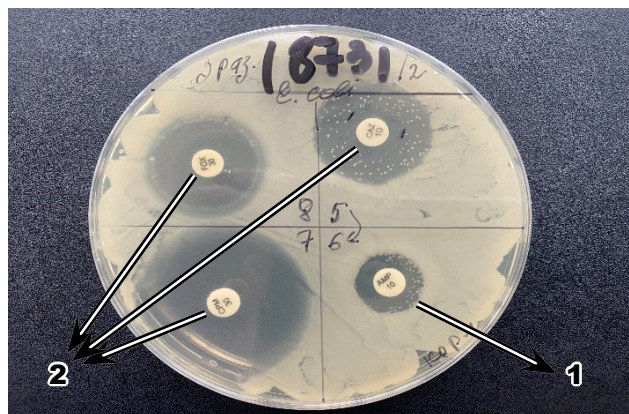


Fig. 2. Formation of antibiotic resistance in individual bacteria of the sensitive population of the *E. coli* strain Ec31 isolated from sunflower meal sample

Note. 1 — the zone of growth inhibition is clear of colony growth (culture is sensitive to the antibiotic); 2 — growth characteristics of individual colonies after exposure to fluoroquinolones and other antibiotics in the zone of inhibition of the sensitive population of escherichia (formation of resistance).

these antibiotics [10, 15]. That is why fluoroquinolones are considered to be priority antibiotics to which resistance in pathogens of bacterial etiology develops to a lesser extent. This is also reported by practitioners and scientists who use fluoroquinolones in severe cases of pneumonia caused by various pathogens, including representatives of the *Enterobacteriaceae* family — *E. coli* and *Klebsiella* spp. [13]. There is still no consensus on this issue. Other researchers and medical practitioners report a low therapeutic level of III and IV generation fluoroquinolones and the need for fluoroquinolones with higher bactericidal activity [11].

The results of our studies have shown the prevalence of fluoroquinolone-resistant *E. coli* strains isolated from different types of animal and poultry feed, which indicates possible risks of disease in animals and poultry and epidemiological consequences due to the possibility of transmission of such resistance to other species of microorganisms and the normal intestinal biota of humans, animals and poultry.

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Фторхінолонрезистентні штами *Escherichia coli* в кормах для тварин і птиці з кормовиробничих підприємств України

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У статті представлено результати вивчення чутливості штамів *Escherichia coli*, виділених зі зразків кормів для тварин і птиці, до фторхінолонів II (офлоксацину, норфлоксацину), III (левофлоксацину) та IV (моксифлоксацину) поколінь. Показано, що високу стійкість до норфлоксацину виявлено в семи штамів виділених ешерихій, що становить 33,3 % ідентифікованих. До офлоксацину та до представника III покоління фторхінолонів — левофлоксацину — були стійкими по три виділені штами *E. coli*, що становить по 14,3 % усіх досліджених. До представника IV покоління фторхінолонів — моксифлоксацину — резистентність виявлено в одного штаму *E. coli*, тобто в 4,8 % досліджених. Результати проведених досліджень з вивчення стійкості до фторхінолонів виділених штамів *E. coli* вказують на значну контамінацію кормів для тварин і птиці фторхінолонрезистентними ешерихіями. Це створює потенціальні ризики з розповсюдження та ймовірної передачі такої стійкості іншим видам бактерій і нормобіоті шлунково-кишкового тракту тварин і птиці при потрапленні в організм з кормом, а також людини — якщо вона вживатиме продукцію тваринного походження після згодовування таких кормів.

Ключові слова: *Escherichia coli*, фторхінолони, комбікорм, висівки, шрот, премікси, мука тваринного походження, рибна мука