

Antimicrobial resistance of Escherichia coli in broilers with colibacillosis in Morocco

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Abstract

Antimicrobial resistance is a worldwide problem in veterinary medicine. Due to the extensive use of antimicrobials, resistance is very common in farm animals. Antimicrobial resistance in *E. coli* from broilers was studied previously in Morocco. The goal of the present study was to determine the prevalence of resistance and to compare the findings with two earlier studies. From 66 different broiler farms, in the region Rabat-Salé-Zemmour-Zaer in Morocco, samples were collected; of these, 61 were tested. In these 61 samples, high prevalence of antimicrobial resistance for colistin (3% resistant), fosfomycin (15% resistant), gentamicin (25% resistant), florfenicol (51% resistant), trimethoprim-sulfamethoxazol (68% resistant), enrofloxacin (87% resistant), amoxicillin (75% resistant), and doxycycline (100% resistant) was demonstrated. Also an increase in prevalence of resistance of some antimicrobials in *E. coli* was found in comparison with two previous studies.

Keywords: *Escherichia coli*, colibacillosis, antimicrobial resistance, broilers, Morocco

Introduction

Colibacillosis is considered the most important infectious disease in poultry. Colibacillosis is caused by *Escherichia coli*, a gram-negative rod of the family of *Enterobacteriaceae* which grows facultative anaerobic and non-pathogenic strains are commensals of the digestive tract. Pathogenic strains like Avian Pathogenic *Escherichia Coli* (APEC) may cause disease in chickens. Colibacillosis occurs in many forms, e.g. swollen head syndrome, yolk sac infection, coligranuloma and colisepticaemia. Colisepticaemia is the most prevalent form of colibacillosis, the respiratory-origin colisepticaemia being the most common type. (Saif, Fadly et al. 2008, Persoons, Callens et al. 2011, Lister, Barrow 2008)

Risk factors influencing the occurrence of respiratory-origin colisepticaemia, are primary respiratory infections caused by e.g. *Mycoplasma* spp., or viral infections (both wildtype and vaccine strains), unfavorable housing conditions like high air concentrations of ammonia, overcrowding and high challenge dose. (Lutful Kabir 2010)

Colisepticaemia is most often seen in birds of 2 to 12 weeks of age. First sign of the disease is a decrease in feed intake followed by depression of the animals and in the progression of the disease animals may also show respiratory signs e.g. high breathing frequencies and gasping. Morbidity and mortality are highly variable. (Lister, Barrow 2008, Saif, Fadly et al. 2008, Persoons, Callens et al. 2011)

Antimicrobials are widely used in the therapy of colibacillosis in poultry worldwide. Due to the extensive use of antimicrobials in poultry and farm animals in general, antimicrobial resistance is a very common in bacteria isolated from farm animals. In the broiler industry this practice has allowed for the development of bacteria harboring multiple antimicrobial resistance. In Morocco, *Filali et al.* and *Amara et al.* demonstrated the prevalence of antimicrobial resistance in broiler chickens diagnosed with colibacillosis. (Filali, Bell et al. 1988, Amara, Ziani et al. 1995)

Antimicrobial resistance is not only a risk for poultry, but also for public health. *Cohen et al.* showed that 48.4% of retail poultry meat in Morocco was contaminated with *E. coli* and 25% of chicken meat contained more bacteria than the maximum established by the Moroccan regulatory standards. Most of the slaughtering of poultry takes place in traditional shops in which hygienic standard are very

low.(Cohen, Ennaji et al. 2007) Research in the Netherlands by *Levestein-van Hall et al.* demonstrated contamination by ESBL-producing *E. coli* in 94 % of the retail chicken meat. A study of *Overdevest et al.* suggests a relationship between retail chicken meat contaminated with drug-resistant bacteria and the appearance of ESBL-genes in the bacterial flora of humans in the Netherlands.(Leverstein-van Hall, Dierikx et al. 2011, Overdevest, Willemsen et al. 2011)

Resistant bacteria contaminating poultry meat may not only stem from the pathogenic *E. coli* carrying genes encoding for antimicrobial resistance, but may also be of the commensal gut flora after the transfer of these resistance genes.(Hart, Heuzenroeder et al. 2006)

In this study antimicrobial resistance in pathogenic *E. coli* in Morocco was evaluated and compared with the situation in 1995 and 1988.(Filali, Bell et al. 1988, Amara, Ziani et al. 1995)

Materials and methods

For this study 66 samples taken from flocks from 66 different farms of broiler chickens in the region of Rabat-Salé-Zemmour-Zaer, were examined. Dead chicks suspected of colibacillosis were dissected. When colibacillosis was confirmed as the cause of death, these chicks were used for further research. The heart, bone marrow, liver, lungs and spleen were selected if these presented lesions, according to *Lister and Barrow* and *Saif et al.*, as a consequence of an infection of colibacillosis.(Lister, Barrow 2008, Saif, Fadly et al. 2008)

The samples were collected from the organs with a sterilized inoculation loop, inoculated on *eosine methylene blue (EMB) agar* plates, see *figure 1*, and incubated for 24 hours at 37 °C.(Leininger, Roberson et al.) Colonies of lactose-fermenting bacteria on EMB agar show a green metallic sheen, see *figure 1*. *E. coli* shares this property with few other species, e.g. *Yersinia enterocolitica* also produces this metallic sheen.(Koneman, Allen et al. 1983) To assist in identifying *E. coli*, chemical identification using *ID 32 E* (bioMérieux), was performed (*figure 2*). *ID 32 E* is a standardized system for identification of *Enterobacteriaceae* and other non-fastidious Gram-negative rods in 24 hours.

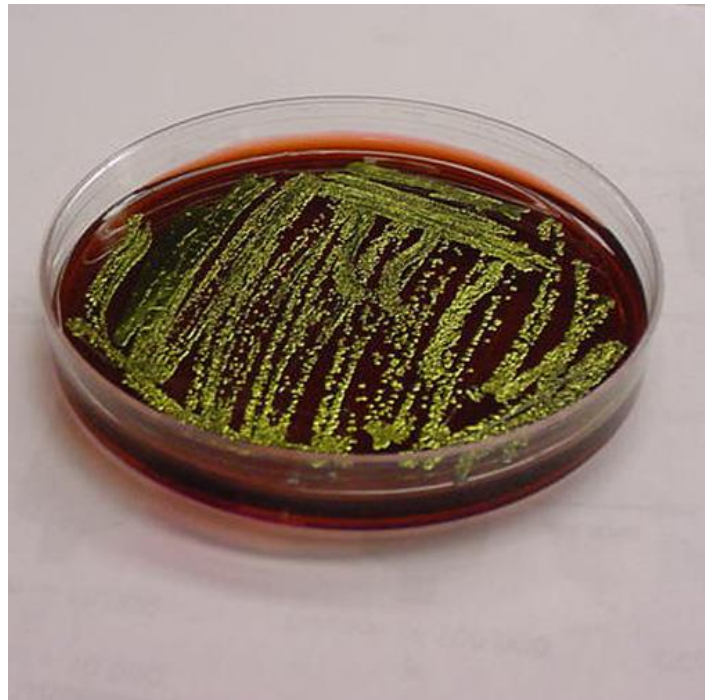


Figure 1: EMB agar (source: www.researchgate.net/post/What_is_the_colour_of_E_coli_colonies_on_EMB_agar)

Not all strains of *E. coli* produce a green metallic sheen, in this study *E. coli* colonies of this type were not examined. Also colonies that were not identified as *E. coli* by *ID 32 E* were not examined.

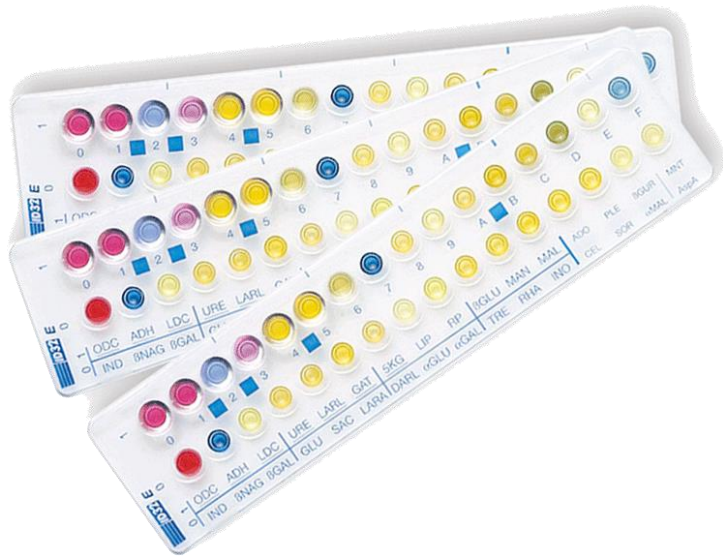


Figure 2: ID 32 E (source: www.biomerieux-industry.com/food/api-id32-microbial-identification)

For the antibiograms and the ID 32 E testing, for each sample a suspension was made by adding green metallic colonies to 5 ml of API NaCl 0.85% medium, to a density visually compared to 0.5 McFarland Standard. This suspension was inoculated by a sterilized cotton swab on Mueller-Hinton plates, on which the antibiotic diffusion discs were placed and incubated for 24 hour at 37 °C. The antibiograms and the ID 32 E were incubated simultaneously and the samples that were not identified as *E. coli*, were not included in the results. The inhibition zone was read according to the manufacturers' instructions and the result was represented as sensitive, intermediate or resistant.

For this study the following antimicrobials were selected: *colistin*, *fosfomycin*, *gentamicin*, *florfenicol*, *trimethoprim-sulphamethoxazol*, *enrofloxacin*, *amoxicillin*, and *doxycycline*.

Results

Resistance

Five samples out of 66 samples were not taken into account, because these were not tested as *E. coli* colonies by ID 32 E. Not all samples were tested for resistance for gentamicin, TMPS or florfenicol, due to the lack of antimicrobial discs. For colistin 61 samples were tested, for gentamicin 52 samples, for florfenicol 59 samples, for fosfomycin 61 samples, for TMPS 60 samples, for enrofloxacin 61 samples, for doxycycline 61 samples and for amoxicillin 61 samples. The percentages of the results are based on the number of samples tested. In *table 1* the prevalence of resistance is shown.

Table 1: resistance; percentages and (numbers of samples found)

Antimicrobial	Number of samples	Sensitive	Intermediate	Resistant
Colistin	61	96.7% (59)	0.0% (0)	3.3% (2)
Fosfomycin	61	83.6% (51)	1.6% (1)	14.8% (9)
Gentamicin	52	71.2% (37)	3.9% (2)	25.0% (13)
Florfenicol	59	49.2% (29)	0.0% (0)	50.9% (30)
TMPS	60	25.0% (15)	6.7% (4)	68.3% (41)
Enrofloxacin	61	11.5% (7)	1.6% (1)	86.9% (53)
Amoxicillin	61	14.8% (9)	9.8% (6)	75.4% (46)
Doxycycline	61	0.0% (0)	0.0% (0)	100.0% (61)

Multiresistance

The results show that most samples resistance to at least two antimicrobials was found. An overview of this multiresistance (varying from 2 to 8 antimicrobials) and the percentage of the samples are shown in *table 2*.

Table 2: multiresistance; number and percentage of samples presenting resistance to 1-8 antimicrobials

Number of antimicrobials	Number of resistant samples	Percentage of resistant samples
1	2	3.3%
2	8	13.1%
3	12	19.7%
4	10	16.4%
5	18	29.5%
6	8	13.1%
7	1	1.6%
8	2	3.3%
Total	61	100%

In most samples resistance to more than one antimicrobial was found. The combinations of antimicrobial resistance are shown in *table 3*.

Table 3; combinations of resistance; colistin (Co), gentamicin (Ge), florfenicol (Fl), fosfomycin (Fo), TMPS (TS), enrofloxacin (En), doxycycline (Do) and amoxicillin (Am)

Combination of antimicrobials	Percentage (number)
Fl.Do	1,6% (1)
Fl.Do.Am	1,6% (1)
Fl.TS.Do.Am	1,6% (1)
Ge.Fl.Fo.TS.En.Do.Am	1,6% (1)
Ge.TS.En.Do.Am	1,6% (1)
Ts.Do	1,6% (1)
Co.Ge.Fl.Fo.TS.En.Do.Am	3,3% (2)
Do	3,3% (2)
Do.Am	3,3% (2)
Fl.En.Do.Am	3,3% (2)
Ge.En.Do	3,3% (2)
Ge.Fl.TS.En.Do	3,3% (2)
Fl.Fo.TS.En.Do.Am	4,9% (3)
Fo.TS.En.Do.Am	4,9% (3)
TS.En.Do	4,9% (3)
En.Do	6,6% (4)
En.Do.Am	8,2% (5)
Ge.Fl.TS.En.Do.Am	8,2% (5)
TS.En.Do.Am	13,1% (8)
Fl.TS.En.Do.Am	19,7% (12)
Total	100.0% (61)

Table 4; prevalence of resistance in the studies of Filali et al. 1988, Amara et al. 1995 and the present study

Antimicrobial	1988 (62 samples)	1995 (258 samples)	2012-2013 (61 samples)
Colistin	0.0%	0.4%	3.3%
Fosfomycin	ND	ND	14.8%
Gentamicin	0.0%	7.0%	25.0%
Florfenicol	ND	ND	50.8%
Chloramphenicol	42.0%	41.0%	ND
Trimethoprim-sulphamethoxazole	8%	61.0%	68.3%
Enrofloxacin	ND	23.0%	86.9%
Doxycycline	ND	ND	100.0%
Oxytetracycline	82.0%	65.0%	ND
Amoxicillin	ND	ND	75.4%
Ampicillin	20.0%	14.0%	ND

Discussion

In this study the cultures examined, were not axenic. More than one colony from the EMB agar plates were suspended to establish the desired visual density of the suspension. Thus samples that were not identified as *E. coli* by ID 32 E could have been discarded incorrectly because these samples were not axenic. Also the colonies that did not show a green metallic sheen which were not used for further research could have been *E. coli*. It is possible that *E. coli* was omitted on false grounds. Also the use of axenic cultures may cause an overestimation with respect to multiresistance.

An overall increase in antimicrobial resistance was found in this study, in comparison to studies performed earlier by Filali et al. and Amara et al. A striking result is from doxycycline, with 100% prevalence of resistance. Besides to doxycycline, high prevalence of resistance to enrofloxacin, amoxicillin, TMPS and florfenicol, was found. To gentamicin and fosfomycin a lower prevalence of resistance was found. An indication of resistance to colistin was found in only two samples, albeit that these two samples showed multiresistance to eight antimicrobials.

Colistin is a small-spectrum antibiotic which is active against gram-negative bacteria by binding to the lipopolysaccharide of the outer cell-membrane. Colistin is not absorbed from the intestines and therefore treatment with colistin will not cure systemic colibacillosis in diseased broilers.(Giguère, Prescott et al. 2007) However, infectious pressure from intestinal sensitive *E. coli* will be reduced. Colistin is essential to human healthcare as a last-resort treatment of certain pneumonias.(Gezondheidsraad 2011) The diffusion test as applied in this study tends to overestimate resistance to colistin due to poor diffusion. So resistance which is shown in this study should be taken with caution, as research has shown that the disk diffusion test is unreliable for testing colistin resistance in bacteria.(Tan, Ng 2006, Galani, Kontopidou et al. 2008)

An intermediate prevalence of resistance to gentamicin and fosfomycin is demonstrated. 25% of the samples appeared resistant to gentamicin. The resistance has increased in comparison to the two previous studies of Filali et al. and Amara et al. Fosfomycin is known for its activity against *E. coli* and was studied for its efficacy in the treatment of colibacillosis.(Giguère, Prescott et al. 2007, Fernandez, Lara et al. 1998, Fernández, Lara et al. 2002) Fosfomycin was not tested by Filali et al. or Amara et al., but it has been used in Morocco for the treatment of colibacillosis. This study shows 14.8% of the samples being resistant to fosfomycin.

High prevalence of resistance was demonstrated to florfenicol, TMPS, enrofloxacin, amoxicillin and doxycycline. Resistance to florfenicol was tested rather than to chloramphenicol as studied by Filali et al. and Amara et al. Chloramphenicol was banned for use in food producing animals because it may cause fatal aplastic anemia in humans. Florfenicol is less sensitive to development of resistance by chloramphenicol acetyltransferase produced by bacteria than chloramphenicol.(Giguère, Prescott et al. 2007) This study shows 68.3% prevalence of resistance to trimethoprim-sulfamethoxazole. This is a slight increase in comparison to 1995, see *table 4*. This is comparable with the situation in Spain and in the United Kingdom according to a study by De Jong et al.(De jong, Thomas et al. 2012) Resistance to enrofloxacin increased dramatically compared to the study of Amara et al. This may be due to the extended use of enrofloxacin to treat colibacillosis in broilers. *Van Boven et al. 2003* postulates that

repeated use of enrofloxacin does not cause high prevalence of resistance in *E. coli*. However in this study it is demonstrated that the prevalence of resistance to enrofloxacin which appeared on the Moroccan market in 1990 has increased to nearly 90% of the isolates tested.(van Boven, Veldman et al. 2003, Amara, Ziani et al. 1995) 75.4% of the samples were resistant to amoxicillin which is according to the widespread prevalence of resistance of *E. coli* to extended-spectrum penicillins.(Giguère, Prescott et al. 2007, De Jong, Thomas et al. 2012) The prevalences of resistance to doxycycline, enrofloxacin and amoxicillin are very high. Every sample used in this research, was resistant to doxycycline. Resistance to tetracyclines is not a surprise, due to the widespread use as a growth promotor in the past.(Marshall, Levy 2011)

The prevalence of resistance in this study was compared with the findings in the studies by Filali et al. and Amara et al., shown in *table 4*. Some antimicrobials differ from the ones used by Filali et al. and Amara et al., these include amoxicillin (ampicillin), doxycycline (oxytetracyclin) and florfenicol (chloramphenicol). This makes a full comparison impossible, but shows high levels of resistance in these classes of antimicrobials. Fosfomycin was not studied previously by Filali et al. and Amara et al.. Enrofloxacin was studied by Amara et al. but not by Filali et al. because it was not available at the Moroccan market at that time.(Amara, Ziani et al. 1995) The increase of the prevalence of resistance to gentamicin compared to the previous studies is a reason for concern, especially when this increase persists in the future. With respect to TMPS, in 1995 an intermediate high prevalence of resistance was found; the increase to nearly 70% which was demonstrated in this study, is a reason for concern. Between this study and the two previous studies some differences exist which make a full comparison difficult. There were different numbers of samples, and Amara et al. took samples from 6 provinces in Morocco, whilst Filali et al. and the present authors took samples from the region Rabat-Salé-Zemmour-Zaer. However, the figures presented may give an indication of the rapid development of resistance and the emergence of multiresistant strains of *E. coli*.

In this study 20 patterns of combinations of antimicrobial resistance were found. Most frequent, 12 samples, was the combination of resistance to florfenicol, TMPS, enrofloxacin, doxycycline and amoxicillin. This is not surprising, since for most isolates resistance was demonstrated to these five antimicrobials. Remarkably, resistance to enrofloxacin appears in all samples resistant to five or more antimicrobials. Resistance to enrofloxacin thus may be related to multiresistance.

As antimicrobial resistance is inherently connected to the use of antimicrobials in the treatment of bacterial infections, the finding of antimicrobial resistance in the 61 samples is not surprising. (Boerlin, White 2007) Interesting are the high prevalence of resistance per antimicrobial and the high prevalence of multi-resistance (18 % resistant to ≥ 6 antimicrobials) in the samples. This forms a threat to the effective treatment of Colibacillosis in Morocco. Also noteworthy is the potential risk formed by resistant bacterial strains for public health in Morocco, as poultry meat consumption has risen in the last thirty years. Poultry meat still is retailed from the traditional slaughtering units which are a potential hazard due to the lack of sanitary utilities.(Ait El Mekki, Jaafari et al. 2006) The results of this study emphasize the need for surveillance of antimicrobial resistance on a regular base and may also urge for an alternative to antimicrobial treatment for Colibacillosis.

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