

**INVESTIGATION OF THE SENSITIVITY OF *E. COLI* STRAINS ISOLATED FROM DOMESTIC ANIMALS TO ANTIBIOTICS AND HEMIO THERAPEUTICS *IN VITRO***

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*Resistance to antibiotics is not a modern phenomenon. On the contrary, penicillin resistance in some bacterial strains developed quickly after its introduction into daily practice. At the same time some bacterial strains developed resistance to almost all known antibiotics, vancomycin included. Vancomycin was for a long time the only efficient antibiotic against staphylococcal infections. It is of special concern the fact that antibiotics are in everyday exploitation in agriculture and veterinary clinical practice which use them not only as a mean of therapeutic treatment, but as an additive in animal feedstuffs in order to promote growth and prevent bacterial infections. The same antibiotics are used in human medicine, which is a persistent problem. In such a way it is possible to develop resistance which can be transferred to human pathogenic bacteria via mobile genetic elements. The incidence of resistant bacterial strains increases year after year not only on a local level, but on a global scale, as well.*

*Monitoring of the use of antibiotics and chemotherapeutics in the Republic of Serbia is not established as such, our intention was to study a number of bacteria isolated from cattle, pigs, poultry, dogs and cats. At this time we are presenting the results for pathogenic strains of *E. coli* in order to determine the use of antibiotics and chemotherapeutics of the old and new generations in domestic animals.*

**E. coli* sensitivity was investigated with the disc diffusion test for: ampicillin, amoxicillin with clavulanic acid, tetracycline, chloramphenicol, gentamicin, and ceftriaxon, sulphamethoxazole with trimethoprim, ciprofloxacin and florfenicol. *E. coli* strains resistant to three or more antibiotics were tested by means of agar dilution method for ciprofloxacin, tetracycline, chloramphenicol, gentamicin and amoxicillin with clavulanic acid by determination of the minimal inhibitory concentration (MIC).*

*The tested *E. coli* strains resulted resistant to all antibiotics and chemotherapeutics with the exception of ceftriaxon and florfenicol. The highest resistance incidence (87.5%) was to tetracycline in *E. coli* strains isolated from pigs, 60% for *E. coli* strains isolated from cattle, 56% isolated from poultry and 20% originating from dogs. *E. coli* strains*

*isolated from cats were sensitive to tetracycline. The highest incidence of ampicillin resistance was determined for E. coli strains originated from poultry (78%).*

*Key words: animals, antibiotics, E. coli, resistance, sensitivity*

## INTRODUCTION

Before the discovery of antibiotics and chemotherapeutics a number of infectious diseases were incurable and mortality in the human population was enormous. This is confirmed by a number of examples from the past. This was a world in which the probability to succumb to infectious diseases before reaching sexual maturity was over 40%, and woman died after childbirth from infections which today are easily cured (Cohen, 2000).

After penicillin was accidentally discovered in 1929 by Alexander Fleming a revolutionary step forward in the treatment of bacterial infections was achieved. Since then the interest of scientist was aimed to discover new antibiotics.

However, soon after the introduction of antibiotics scientists have noticed the appearance of some bacterial strains which do not respond to antibiotic treatment (Cohen, 2000; Fluit and Schmitz, 2004). The widespread of resistant bacteria is amplified by the use of antibiotics as feed additives and growth promoters and for the prevention of bacterial infections (Franklin, 1999). The incidence of bacteria resistant to antibiotics increases year after year both on a local and global level and is aimed towards a growing number of antibiotic drugs (Spratt, 1994; Livermore, 2004). A number of authors (Wray *et al.*, 1993) are of the opinion that multiple resistance is present in the majority of *E. coli* strains isolated from domestic animals, especially calves suffering from colibacillosis. In some strains bacteria are resistant for a majority of antibiotics, thus resulting in a small number of efficient antibiotics and chemotherapeutics. Das (1984) has disclosed bacterial sensitivity to amikacin of all *E. coli* strains isolated from pigs. Takahashi *et al.* (1990) have described the sensitivity of ofloxacin in a number of *E. coli* strains isolated from pigs, cattle and poultry. A number of authors are of the opinion that in order to obtain an efficient therapeutic effect in diseased animals new antimicrobial drugs such as aminotiazolil cephalosporines (cefotaxim and cefkvin) and fluoroquinolones (Orden *et al.*, 1999) should be applied.

Soon after the introduction of florfenicol into veterinary clinical practice in the USA were disclosed the first cases of colibacillosis in calves infected with *E. coli* strains resistant to this antibiotic (Cloeckert *et al.*, 2000). The same resistance was discovered in *E. coli* strains originating from poultry, despite the fact that this antibiotic has never been used in this species (Keyes *et al.*, 2000).

Further studies on *E. coli* resistance to antibiotics have been carried out by Krnjaić *et al.* (2000) and Mišić (2005). As florfenicol is in use in Serbia for almost three years we were motivated to study the sensitivity of *E. coli* to florfenicol compared to other antibiotics. Florfenicol is fluorinated timaphenicole and hloramphenicole derivate which is in use in Serbia in the recent three years.

## MATERIAL AND METHODS

In this study samples of internal organs of deceased animals, feces and swabs from ill animals (cattle, pigs, poultry, dogs and cats) were taken. In order to isolate *E. coli* strains the following nutrition media were used: MacConkey agar (Torlak), base for blood agar (Torlak) with added 5-6% defibrinated sheep blood, Columbia CNA agar (Becton Dickinson nutritious bujon, Torlak), TTC semi liquid agar with added 2,3,5, trifeniil-tetrazolium chloride, Hugh-Leifson media (bioLab), buffered peptone water and triple sugar (Torlak).

For the identification of the isolated strains routine laboratory tests with the following nutritious media and reagents were used: Simmons citrate agar (Torlak), MR/VP bujon (Torlak), Christensen urea agar (Torlak), nutritious gelatine (Torlak), Oxydase reagent dropper (Becton Dickinson), Lysine iron agar (Becton Dickinson), as well as identification systems BBL Crystal Gram positive ID kit and BBL Crystal Entero/nonfermenter ID kit (Becton Dickinson).

Sensitivity studies on the isolated bacteria were completed by the disc diffusion method on Mueller Hinton agar with the use of antibiogram discs (Becton Dickinson and Bioanalyse) and tablets (Torlak) for the following antibiotics: ampicillin, amoxicillin with clavulanic acid, tetracycline, chloramphenicol, gentamicin, ceftriaxon, sulphamethaxasole with trimetoprim, ciprofloxacin and florfenicol. To determine the minimal inhibitory concentration (MIC) by the dilution method pure active ciprofloxacin, tetracycline, chloramphenicol, gentamicin, and amoxicillin with clavulanic acid (Sigma, Torlak and Hemofarm) were used. Referent strain of *Escherichia coli* ATCC 25922 was used as a control.

Readings of the obtained results were performed according to the recommended by Clinical Laboratory Standards Institute (CLSI, 2008). Based upon the inhibition zone radius the tested bacteria strains were grouped into three categories: S (sensitive), I (intermediate) and R (resistant).

## RESULTS

A total of 35 *E. coli* strains of the following provenience were tested: 10 from cattle, 9 from poultry, 8 from pigs, 5 from dogs and 3 from cats. The antibiotics most often used in veterinary practice in Serbia were tested. Selection was based upon the fact that their are listed for treatment of infections caused by *Enterobacteriae* and are recommended by National Committee on Clinical Laboratory Standards, NCCLS (2004) and CLSI (2008).

Table 1. Presence as well as percentage of *E. coli* strains resistant to tested antibiotics

Antibiotic tested	Amp	Akk	Tet	Chl	Gen	C 3	Sxt	Cip	Ffc
Total number of resistant strains	16	6	19	10	4	0	12	4	0
Percentage of resistant strains (%)	45.71	17.41	54.29	28.57	11.43	0.00	34.29	11.43	0.00

Table 1 shows that all isolated *E. coli* strains are resistant to all tested antibiotics with the exception of ceftriaxon and florfenicol. Graph 1 and Table 2 describes the resistance to antibiotics in *E.coli* isolated from all animal species included in the study.

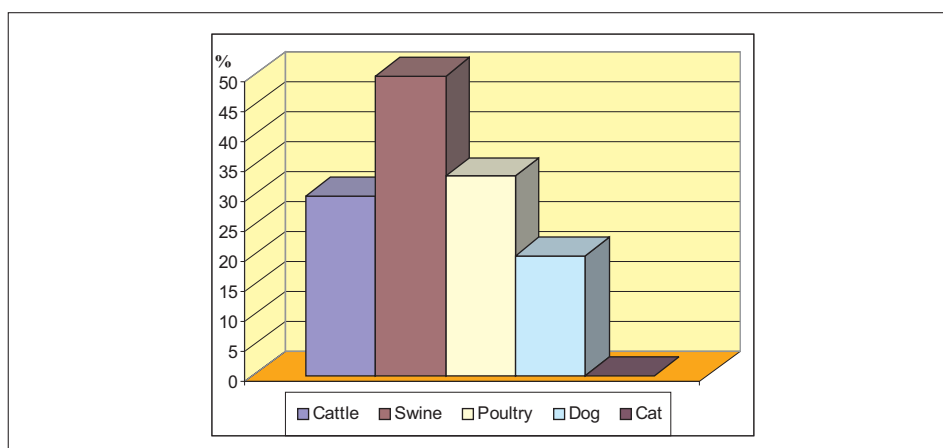


Figure 1. Percentage of resistant strains *E. coli* isolated from certain animal species

Table 2. Presence, as well as percentage, of the resistance of *E. coli* strains isolated from different animal species

Animal species		Amp	Akk	Tet	Chl	Gen	C 3	Sxt	Cip	Ffc
Cattle	Number	4	1	6	6	1	0	5	0	0
	%	40.00	10.00	60.00	60.00	10.00	0.00	50.00	0.00	0.00
Swine	Number	3	0	7	2	2	0	3	1	0
	%	37.50	0.00	87.50	25.00	25.00	0.00	37.50	12.50	0.00
Poultry	Number	7	3	5	1	0	0	3	2	0
	%	77.78	33.33	55.56	11.11	0.00	0.00	33.33	22.22	0.00
Dogs	Number	2	1	1	1	1	0	1	1	0
	%	40.00	20.00	20.00	20.00	20.00	0.00	20.00	20.00	0.00
Cats	Number	0	1	0	0	0	0	0	0	0
	%	0.00	33.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00

The highest percentage (78%) of resistance to ampicillin was in *E. coli* isolated from poultry, 40% strains isolated from dogs and cattle and 37.5% isolated from pigs. It is of interest that no strains isolated from cats were resistant to ampicillin. Resistance to amoxicillin with clavulanic acid was registered in 10% *E. coli* strains isolated from cattle, 20% isolated from dogs 33% from pigs and poultry while all strains isolated from cats were amoxicillin sensitive.

Tetracycline *E. coli* sensitivity was in strains isolated from pigs (87.5%) cattle (60%), poultry (55%), dogs (20%) and samples taken from cats were all sensitive

to this antibiotic. Equally, no resistant *E. coli* strains in cats were described for chloramphenicol and in cattle were resistant in 60% samples, pigs 25%, dogs 20% and poultry 11%. No resistance to gentamicin was recorded in samples taken from cats and poultry. Strains taken from pigs were resistant in 25%, from dogs 20% and cattle 10%. In the tested *E. coli* strains there was no resistance to ceftriaxone and florfenicol.

Resistance to sulphamethoxazole + trimethoprim was present in 50% strains from cattle, 37.5% pigs, 33% poultry and 20% strains originating from dogs. *E. coli* strains were resistant to ciprofloxacin in 22% taken from poultry, 20% from dogs, 12.5% from pigs and no resistance was recorded for strains taken from cats or cattle.

Besides the determination of *E. coli* resistance to antibiotics further studies were carried out in order to determine the presence of multiresistance to three or more antibiotics. Results are shown in Table 3.

Table 3. Percentage of the multiresistant *E. coli* strains as a proportion of the total strains tested

Animal species	Multiresistant strains (%)	Resistance to the number of antibiotics (%)					
		3	4	5	6	7	8
Cattle	30.00	10.00	20.00	0	0	0	0
Swine	50.00	25.00	25.00	0	0	0	0
Poultry	33.00	22.22	0	0	11.11	0	0
Dogs	20.00	0	0	0	0	20.00	0
Cats	0.00	0	0	0	0	0	0

One *E. coli* strain (Table 3) isolated from a dog was resistant to 7 antibiotics, and resistance to 6 antibiotics was recorded in a sample taken from poultry. Samples taken from pigs and cattle were resistant to 3 and 4 antibiotics, respectively. Other *E. coli* strains isolated from poultry were resistant to 3 antibiotics.

Resistance profiles to individual antibiotics are given in Tables 4, 5 and 6.

Table 4. Resistance of the *E. coli* strains isolated from cattle

Number of antibiotics tested	Resistance	Number of bacterial strains
4	ampicillin, amoxicillin with clavulanic acid, tetracycline, chloramphenicol	1
4	ampicillin, tetracycline, chloramphenicol, sulphamethoxazole + trimethoprim	1
3	ampicillin, tetracycline, gentamicin,	1
2	chloramphenicol, sulphamethoxazole + trimethoprim	4
2	ampicillin, tetracycline	1
1	tetracycline	2
0	No resistance	0

Table 5. Resistance of the *E. coli* strains isolated from swine

Number of antibiotics tested	Resistance	Number of bacterial strains
4	ampicillin, chloramphenicol, ciprofloxacin, sulphamethoxazole + trimethoprim,	1
4	ampicillin, tetracycline, gentamycin, sulphamethoxazole + trimethoprim	1
3	tetracycline, chloramphenicol, sulphamethoxazole + trimethoprim	1
3	ampicillin, tetracycline, gentamicin	1
1	tetracycline	4
0	No resistance	0

Table 6. Resistance of the *E. coli* strains isolated from poultry

Number of antibiotics tested	Resistance	Number of bacterial strains
6	ampicillin, amoxicillin with clavulanic acid, tetracycline, chloramphenicol, sulphamethoxazole + trimethoprim, ciprofloxacin	1
3	ampicillin, amoxicillin with clavulanic acid, tetracycline	1
3	ampicillin, tetracycline, sulphamethoxazole + trimethoprim	1
2	ampicillin, sulphamethoxazole + trimethoprim	1
2	ampicillin, amoxicillin with clavulanic acid	1
1	ampicillin, tetracycline	1
1	ciprofloxacin	1
1	tetracycline	1
0	No resistance	1

## DISCUSSION

*E. coli* are the dominant bacteria present in the digestive system of mammals. Its isolation is relatively easy and rapid due to its short generation interval and specific easily recognizable colonies with a unique IMV and C profile. Due to this *E. coli* is often used in genetic and microbiological *in vitro* studies including studies on the presence, distribution and mode of transfer of bacterial resistance (Nijsten *et al.*, 1996; Livermore, 2004). Moreover, the presence of resistance of some *E. coli* strains is a good indicator of the use of antibiotics in domestic animals.

One of the specificities of this study was the investigation of resistance to florfenicol of some *E. coli* strains. Florfenicol has been approved for veterinary use

in USA in 1996, but only for the treatment of cattle respiratory infections caused by *Pasteurella* spp. Soon after the introduction of florfenicol into veterinary practice the first cases of colibacillosis resistant to florfenicol have been described in calves (Cloeckaert *et al.*, 2000). The same type of resistance was discovered in poultry, despite the fact that this drug has never been used in this animal species (Keyes *et al.*, 2000).

The gene responsible for *E. coli* resistance to florfenicol is described as the *Flo* gene and has been isolated for the first time from *Pasteurella piscicida* isolated from fish skin. The *Flo* gene is transferred not only via plasmids, but vertically by chromosome division, as well. The *Flo* gene codes resistance not only to florfenicol, but to chloramphenicol, also (Peterson, 2006). Bacteria in which a primary resistance to chloramphenicol was described can be sensitive to florfenicol. It can be concluded that resistance to phenicol antibiotics there is a number of different resistance genes which are transferred within the bacterial populations and are responsible for encoding different mechanisms of resistance to these antibiotics.

Some authors are of the opinion that even before florfenicol has been introduced into practice a gene responsible for resistance was already present (Gavrović, 2007). Such a belief was supported by the report of a study carried out in USA (1996) on a number of poultry farms on which samples from diseased birds were collected. A total of 4% of all isolated *E. coli* strains resistance to florfenicol was recorded despite the fact that this antibiotic has never been used in poultry farming (Keyes *et al.*, 2000).

On the other hand there are reports that *flo* gene has appeared more than 20 years ago when florfenicol was introduced for limited use in aquaculture. In our study we have not recorded a single case of *E. coli* resistance to florfenicol. This report is in agreement with the findings of Filipović (2005) for the territory of Montenegro. For the moment these are the only two clinical veterinary microbiology studies on *E. coli* florfenicol resistance conducted on the territory of former SRJ. It is interesting to note that even if florfenicol has been in use for almost three years in veterinary practice in Serbia resistance to this antibiotic has not yet been recorded. However, in the USA resistance to this antibiotic has been recorded the very same year it has been introduced into veterinary practice. Based upon these facts it could be concluded that florfenicol is scarcely used in Serbia, but according to statistical data this antibiotic is very popular and intensively used in all epizootiological areas in Serbia and other neighboring countries.

It has been confirmed that florfenicol has no harmful effects on humans, but still it is not in use in human clinical practice. Three pharmaceutical forms of this drug are approved for veterinary practice: injections, per os solutions and feed supplements. Florfenicol is in use for almost 10 years but up to now on the market there are no antibiogram discs or tablets for routine use in *in vitro* studies.

As far as *E. coli* resistance to other antibiotics results obtained in this study differ from previously published data for the territory of ex SRJ. Krnjaić (2000) reported that 85% *E. coli* samples taken from diseased calves and 90% samples from broilers were ampicillin resistant. Our results have described a moderate



resistance to ampicillin. Thus, *E. coli* strains originating from cattle and calves with diarrhea were 40% resistant and strains originating from poultry were resistant in 78% samples. Even lower resistance prevalence (40%) in poultry *E. coli* strains was recorded by Mišić (2005). The lowest resistance recorded in this area (20%) in poultry *E. coli* strains was documented by Mišić and Filipović (2005) in poultry samples from Montenegro.

According to the results published by Krnjaić (2000) the presence of *E. coli* resistant to amoxicillin and clavulanic acid in sick calves (80%) and chicken (90%) can be considered to be high. In the reported study we have reported 10% *E. coli* isolated from cattle (and affected calves) to be resistant to amoxicillin and clavulonic acid. Studies carried out by Mišić and Filipović (2005) show an *E. coli* resistance to amoxicillin and clavulanic acid as low as 21% in strains originating from calves.

During our study we have isolated 33% *E. coli* strains from poultry which were resistant to amoxicillin and clavulanic acid. These results differ from those reported by Mišić (2005) and Filipović (2005) who did not register *E. coli* resistance to amoxicillin and clavulanic acid. Our results are close to those reported by Mišić (2005) and Filipović (2005), as resistance to amoxicillin and clavulanic acid in *E. coli* strains isolated from pigs was not registered, thus being in accordance to the results published by Krnjaić (2000) and Mišić (2005). Filipović (2005) reported resistance to amoxicillin and clavulonic acid in 12.5% *E. coli* strains isolated from pigs in Montenegro.

Our results are in agreement with the findings published by Krnjaić, Filipović and Mišić who reported that *E. coli* resistance to ceftriaxon was not recorded in any of the samples isolated from cattle, broilers, pigs, dogs and cats. A significant difference for resistance was reported for chloramphenicol. Krnjaić (2000) accounted for a resistance to chloramphenicol in the range from 5 to 55% in *E. coli* strains isolated from all sampled animals. According to our findings the prevalence of resistance in *E. coli* strains originating from poultry was 11%, pigs 25% and cattle 60% which is a high percentage bearing in mind that chloramphenicol is banned for use in cattle. A very high prevalence of resistance was recorded for chloramphenicol in dogs (20%). Samples taken from cats were all sensitive to chloramphenicol. It is interesting to note that these results are somewhat different compared to the findings reported by Mišić (2005) who stated that *E. coli* strains sampled from dogs were in 50% cases resistant to chloramphenicol and all samples collected from poultry were chloramphenicol sensitive. Filipović (2005) reported *E. coli* resistance in 50% samples collected from dogs, however their results are comparable to the previously reported.

By further analysis and comparison of the obtained results with the results reported by Krnjaić (2000) the prevalence of resistance to sulphonamides in piglets and poultry ranged from 70% to 95% which is higher than the results reported in this paper (33% to 38%). The highest prevalence in our study was 50% in cattle, but in cats was 0% which is very interesting when we are aware of the fact that sulpha- preparations are widely used in small animal practice. Similar results were reported by Mišić (2005) and Filipović (2005) with the difference that Mišić



reported 33% cases of *E. coli* strains sampled from cats were resistant to sulphametoxazol + trimethoprim.

Regardless of species, age and health status *E. coli* resistance to tetracyclines was not reported in cats. However, resistance to tetracyclines was reported in 20% *E. coli* strains isolated from dogs, 56% from poultry and 60% from cattle which does not match the results obtained in other countries where a *E. coli* tetracycline resistance of over 90% was reported. Such results were to be expected. The only coincidence for the results published by authors from ex SRJ, as well as from other regions, are regard tetracycline resistance of *E. coli* strains sampled from pigs, which in this study was reported to be 87%. It is of significance to stress out that in this study the resistance to gentamicin of *E. coli* sampled from poultry and cats was not recorded. *E. coli* strains sampled from other animal species were resistant to this antibiotic in less than 25% cases.

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### ISPITIVANJE *IN VITRO* OSETLJIVOSTI NA ANTIBIOTIKE I HEMIOTERAPEUTIKE SOJEVA *E. COLI* IZOLOVANIH OD ŽIVOTINJA

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#### SADRŽAJ

Rezistencija na antibiotike nije skorašnji fenomen, naprotiv, rezistencija na penicilin kod nekih sojeva bakterija javila se vrlo brzo posle njegove primene u praksi. Takođe, neki sojevi bakterija postali su rezistentni na praktično sve antibiotike u kliničkoj upotrebi, pa čak i na vankomicin koji je duže vreme bio jedini efikasan antibiotik u lečenju stafilokoknih infekcija. Poseban razlog za zabrinutost predstavlja upotreba antibiotika u poljoprivredi i veterinarskoj kliničkoj praksi jer se osim primene u lečenju životinja, iako su zabranjeni, koriste kao promoteri rasta i u prevenciji bakterijskih infekcija, a isti antibiotici (ili sa istim načinom delovanja na bakterije) primenjuju se i u humanoj medicini. Na taj način je moguće da se neodgovornom upotrebom antibiotika razvije rezistencija koja može znatno brže da se prenese na patogene bakterije ljudi preko mobilnih genetičkih elemenata. Pojava rezistentnih sojeva bakterija se povećava iz godine u godinu, kako na lokalnom, tako i na globalnom nivou.

Kako monitoring korišćenja antibiotika i hemioterapeutika u veterinarskoj medicini u Republici Srbiji nije uspostavljen, a problem rezistencije bakterija na antibakterijske lekove je prisutan, ovim ispitivanjem želeli smo da obuhvatimo više vrsta bakterija izolovanih od goveda, svinja, živine, pasa i mačaka. Rezultate dobijene u ovom ispitivanju iznosimo samo za patogene sojeve *E. coli*, kako bi na indirektan način ustanovili primenu antibiotika i hemioterapeutika starije i novije generacije kod pomenutih životinja. Osetljivost navedenih sojeva *E. coli* ispitivana je na: ampicilin, amoksicilin sa klavulanskom kiselinom, tetraciklin, hloramfenikol, gentamicin, ceftriakson, sulfametoksazol sa trimetoprimom, ciprofloksacin i florfenikol, primenom disk difuzione metode. Kod sojeva *E. coli* kod kojih je primenom disk-difuzione metode ustanovljena rezistencija na tri i više antibiotika, odnosno multi rezistencija dalje ispitivanje je vršeno i dilucionom metodom u

agaru na: ciprofloksacin, tetraciklin, hloramfenikol, gentamicin i amoksicilin sa klavulanskom kiselinom radi utvrđivanja minimalne inhibitorne koncentracije (MIC).

Kod ispitivanih sojeva *E.coli* ustanovljena je rezistencija na sve ispitivane antibiotike i hemioterapeutike, izuzev na ceftriakson i florfenikol. Najveći procenat rezistencije od 87,5% ustanovljen je na tetraciklin kod sojeva *E.coli* izolovanih od svinja, a zatim 60% kod sojeva izolovanih od goveda, od 56% kod sojeva izolovanih od živine i od 20% kod sojeva poreklom od pasa, dok su sojevi *E. coli* poreklom od mačaka bili osetljivi na tetraciklin. Najviši procenat rezistencije na ampicilin od 78% ustanovljen je kod sojeva *E. coli* izolovanih od živine.

