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Characterization of *Escherichia coli* strains isolated from pigs in semi-industrial swine farm importing gilts pigs from western European country

Albena Dimitrova*, Simeon Yordanov and Krasimira Petkova

National Diagnostic Research Veterinary Medical Institute, 1606 Sofia, Bulgaria
**Corresponding author:* aldimas@abv.bg

Abstract

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In a semi-industrial pig farm importing gilts sows from France, where the respiratory disease complex (PRDC) is a problem and was registered enzootic flowing of colibacteriosis (CB) was carried complexes examination on the characteristics of the persistent *E. coli* strains. Three times through 6 months, organ samples (OS) and rectal swabs sample (RSS) were collected from pigs 1-10, 11-20, 21-30 and 31-45 days of age, that were tested bacteriologically for *E. coli.* The received isolates were typified and tested for susceptibility to 17 antimicrobial means.

Isolated were 37 strains of *E. coli* from 7 serogroups, of which O8, O20 and O157 defined as "old" and O74, O136, O148 and O159 as "new". In most cases, the strains were in different combinations but almost always positive for F4 and F5 and less often for F6. Strains of serogroup O8 persisted from the first to the 45th day after birth; – from O20 and O136 – from birth to weaning at 30 days; – from O159 – from 11th to 20th day; – from O157 – from 11th to 45th day; – from O148 – from the 21st day to weaning at 30 days; and from O74 – only after weaning, from the 31st to the 45th day. Isolated *E.coli* strains were sensitive to most of drug means of the groups of aminoglycosides, amphenicoles and fluorinated quinolones as well as to the sulfamethoxazole+trimethoprim combination. In some of them was registered increased sensitivity compared to past periods. Except to beta-lactams, many of the strains are also resistant to tetracyclines, macrolides, streptomycin and colistin.

Key words: pigs; *E. coli;* serogroups; antibiotics; sensitivity

Introduction

The colibacteria (*Escherichia coli*, *E. coli*) have somatic (O), capsular (K), fringer or flagellar (H) and fimbrial (F) antigens. On the basis of the O- and K-antigens, the serogroup of the strain is determined and of the H- and F-antigens – the serovar, which estimates the virulence of the isolate. Those who possess pathogenic potential realized it after colonization of the intestinal tract or after disseminating in other organs and systems. Depending on this, intestinal (diarrheal) and extra-intestinal (non-diarrheal) *E. coli* are distinguished (Okerman & Devrise, 1985; Fairbrother et al., 1994; Francis, 2002; Popova, 2009).

The virulence factors of *E. coli* include fimbriae, capsules, endotoxins and exotoxins. It has been found that in pigs there are 5 antigenic different fimbres types: F4, F5, F6, F18 and F41. It is now known that F18 is not associated with colibacteriosis (CB) in suckling pigs but it is engaged in colienteritis (CE) and endemic disease (ED) after weaning, and F41 is more common in association with F5 and its presence in a naturally exploded CB is not certain (Francis, 2002; Yordanov, 2014; Lyutskanov, 2012). The toxins produced by

enterotoxigenic *E. coli* (ETEC) are labile toxins (LT), Stable are Toxins A (STA), Stable Toxins B (STB) and Verotoxin (Shiga like toxin – SLT-1 and SLT-2). The first three toxins act locally and cause hypersecretion of fluid from the intestine, while verotoxin is responsible for the systemic vascular effects in edema disease (Okerman & Devrise, 1985; Fairbrather et al., 1994; Francis, 2002).

The *E. coli* diarrhea group includes several pathovars that first colonize the intestinal mucosa and then, through various mechanisms, perform specific morphological changes and functional disorders resulting in a clinically manifested diarrheal symptom complex. Depending from this, are distinguished the following pathovars: – 1. Enteropathogenic *E. coli* (EPEC), which does not produce exotoxins; – 2. Enterotoxigenic *E. coli* (ETEC), which, through fimbriae, adheres to mucose of the small intestine and produces LT and ST enterotoxins, causing neonatal diarrhea. – 3. Enterohaemorrhagic *E. coli* (EHEC), producing a shyga-like toxin (SLT) that exhibits cytotoxicity and degrades the vero cells. – 4. Entero-invasive *E. coli* (EIEC) that are not proven in animals. – 5. Enteroagregative *E. coli* (EAgEC) that covers enterocyte and disrupt their function. – 6. Diffuse adherent *E. coli* (DAEC), which adhere to the entire surface of enterocytes and block their function (Fairbrather et al., 1994, Francis, 2002, Lyutskanov & Urumova, 2010, Lyutskanov, 2012, Yordanov, 2014).

By studying ETEC isolated from pigs, Francis (2002) grouped virulent determinants around *E. coli* serotypes and the age of naturally infected pigs in a table facilitating the characteristic of isolated wild strains. It is clear that non-hemolytic strains of different "O" groups, F5+ and F6+, produce STA toxin and cause colidiarrhea only in newborns, whereas hemolytic strains, that are F4+ and produce LT, STB and STA enterotoxins, cause gastroenteritis (GE) both in newborn piglets and weaned pigs. The situation is different for the haemolytic strains of O138, O139, O141 and O157 that are F18+ and produce STA, STB and SLT toxins, they do not cause disease in the suckling but cause edematous disease and diarrhea in weaned pigs. It was found that for F6 and F18 the expression *in vitro* is particularly problematic.

At the beginning of the 21st century CB in pigs in Bulgaria is widespread in pig farms of industrial type and causes great economic damages. During the period 2000-2005, Dimitrova et al. (2011a) examined 1446 materials from 16 pig complexes (PC) and pig farms (PF) and found that CE and ED in pigs in many cases were associated with Porcine reproductive and respiratory syndrome (PRRS) and Porcine Circovirus disease (PCVD) and are caused by strains of 10 „O" serogroups, incl. hemolytic and non-hemolytic: O8 (1.6%), O54 (10.6%), O78 (1.1%), O101 (11.7%), O138 (8.5%), O139 (17%), O141 (2.7%), O147 (6.4%), O149 (12.8%) и O157 (27.6%). It is noteworthy that the strains of some groups were recorded in only one calendar year: – O141 in 2002, O8 in 2004 and O78 in 2005. Over the next 5 years (2006-2010), Dragoycheva et al. (2011) examined bacteriologically 335 materials from pigs with diarrhea and in 129 cases (38.5%) were isolated EPEC, ETEC and EHEC strains of 11 serogroups including: – O8, O20, O54, O78, O101, O138 , O139, O141, O147, O149 and O157, as dominating are the strains of group O157 (13.2%), O139 (11.6%), O138 (9.3%) and O147 (9.3%). From a study by Dimitrova et al. (2014) on strains of *E. coli* in an industrial pig complex, it is clear that the non-hemolytic strains, which are F5 + and F6 +, cause alone coliinfections (CI) to the the highest degree in piglets up to 10 days of age, and more rarely, alone or in association with hemolytic strains positive for F4 and F5 in larger suckling and weaned pigs, whereas hemolytic ETEC and EHEC strains that have fimbriae cause CE in both suckling and weaned pigs. Petkova (2017) reported that during the period 2011-2015, CI in pigs were caused by representatives of 12 hemolytic and non-hemolytic serogroups: O8, O9, O20, O25, O74, O78, O136, O138, O139, O148, O149 and O157, as the O157, O149, O20 and O74 strains being dominant. As new, registered in 2014, the strains of O9, O25, O74, O136 and O148 positive for F4 and F5 are accepted. Luppi et al. (2015) through multiplex PCR examined 159 *E. coli* isolates associated with post weaning diarrhea (PWD), finding that 95.24% of them were hemolytic and a prevalence of the F4 genotype was 54.76%, for F18 – 33.33 % and for F6 – 1.19%, as in 5 cases the strains contained more than one fimbrial gene.

In the period 2006-2010, Dimitrova et al. (2011-b) have tested for susceptibility 47 diarrhea strains of *E. coli* to 24 antimicrobial means (AMM) by the disc-diffusion method. It was found sensitivity to: – Florfenicol – 87.5%, Enrofloxacin – 80%, Pefloxacin – 78.7%, Cefamandole – 71.43%, Gentamicin – 68.2%, Flumequine – 62.5% and Kanamycin – 55.56%. The highest resistance is found in: Lincomycin – 100%, Aivlosin – 95.45%, Tetracycline – 87.88%, Tylosin – 80%, Doxycycline – 68.42%, Chlortetracycline – 66.67%, Streptomycin – 60% and Nalidixic acid – 60%. Investigating the susceptibility of *E. coli* strains isolated from pigs in SC with intensive breeding activity, Dimitrova et al. (2014) detected susceptibility to means from the group of aminoglycosides and fluorinated quinolones and multiple resistance to a large part of the another means of the strains isolated in the period until 2011, and to a lesser extent in isolates from 2012 and 2013, which is explained by the replacement of the breeding flock and the character of the new *E. coli* strains. Investigating the sensitivity of intestinal *E. coli* strains iso-

lated from pigs to florfenicol, Yordanov et al. (2016), found that in 2015 the strains showed sensitivity to 12 of the 24 AMM used, as to 7 of them (kanamycin, gentamicin, amikacin, florfenicol, enrofloxacin, pefloxacin and norfloxacin) sensitivity ranging from 57.1% to 85.7% and resistance to 16 of the AMM, incl. to 11 of them in a range of 50 to 100%. Petkova (2017) reported that 62 strains of *E.coli* from suckling and growing pigs isolated during the period 2011 – 2015 were tested for susceptibility to 24 AMM, and found high sensitivity (over 50%) to 8 (33.3%) of them: – amikacin – 92.9%, kanamycin – 73.1%, enrofloxacin – 72.6%, gentamicin, – 71.0, sulfamethoxazole + trimethoprim – 69.8%, norfloxacin – 63.0%, florfenicol – 62.9% and pefloxacin – 61.8%. At the same time, the number of agents to which the test strains were resistant to more than 50% was increased to 10, including: – amoxicillin – 93.9%, ampicillin – 81.2%, oxytetracycline – 75.8%, tiamulin – 73.5%, erythromycin – 70.6%, doxycycline – 55.5%, tylosin – 55.5%, tylmicisin – 53.6% and nalidixic acid – 53.4%, and the resistance to colistin and streptomycin is already close to this limit. According to Mos et al. (2010), the disc diffusion method is reliable in antibiotic testing, easy to perform and inexpensive, and the results are excellent reproducible and comparable to those of the serial dilution agar method.

The purpose of this study was to investigate the character of *E. coli* strains causing colienteritis and edema disease in suckling and weaned pigs in pig farm, importing gilts pigs from Western Europe and to test their sensitivity to used antimicrobials.

Materials and Methods

In a semi-industrial farm with a capacity of 350 sows, importing replacement gilts pigs from France were carried out preliminary out-of-laboratory and laboratory investigations in which the presence of the porcine respiratory disease complex (PRDC) and the enzootic flowing of colibacteriosis were detected. In connection with this, we included vaccinations against PRRS, PCVD, Enzootic pneumonia (EP), *Actinobacillus pleuropneumonia* (APP) and CB in the immunoprophylaxis schedule. The purchased replacement gilts were treated metaphylactically with Feniveix-premix, two 5-day courses, with a 10-day interval, and the pregnant sows entering the premises for farrowing, were injected once with Florfenicol.

Clinical and patho-morphological investigations were performed in 2015-2016. Three times, on 6 months, we took organ samples (OS) – 6 pcs. and rectal swab samples (RSS) – 60, from pigs aged 1-10; 11-20; 21-30 and 31-45 days from birth, which we studied bacteriologically for *E. coli*. Studies included: cultivation in liquid and solid nutrient media; microscopic examination; biochemical identification with semi-automatic CRISTAL system (Becton Dickinson, USA) and serotyping by slide agglutination with saturated agglutinating anti-*E. coli* sera to detect O, F4, F5 and F6 antigens. Evaluation of F18 and exotoxins production was performed by comparison of the O-groups, the presence of hemolytic and F-antigens in the isolated *E. coli* strains according to the Francis classification (2002). The strains tested for susceptibility to 17 AMM from 9 pharmacological groups, using the Bauer-Kirby disc-diffusion method (World Organization for Animal Health, 2004).

Results

In the clinical examinations of the newborn pigs, we found that the letters were multiple and flattened, without premature or late births. Born pigs were vital, with high body weight and were developing normally. Only in separate litter were observed under 3-4 piglets with manifestations of coli enteritis, which treated individually with gentamicin, enrofloxacin and sulfamethoxazole +trimethoprim (SMS + T). On the 30-th day of birth, weaned pigs were in good physical and clinical condition, with rare cases of individual pigs showing signs of CE, which we treated individually with gentamicin and enrofloxacin or the entire batch treated methaphylactically with florfenicol. In the pathomorphological examinations of the internal organs of dead suckling piglets (4 pcs.) and weaned pigs (2 pcs.) with diarrhea, we found changes in the digestive tract and the liver, characteristic of coli infections. In the bacteriological test of 6 pcs. OS and 60 pcs. RSS from suckling and weaned pigs, we isolated 37 pcs. *E. coli* cultures from 7 O-serogroups originating from all age groups of the pigs. In most cases, the isolates were positive for F4 and F5 and rarely for F6. The most strains *E. coli* belonged to serogroups: O8, O157, O136 and O20. It was found that strains of serogroup O8 persist from birth to day 45 and are presented in the first two ten days as non-hemolytic and F5 +, and after then as hemolytic and F4 +; – strains of serogroups O20 and O136 persist from birth to weaning at day 30 and are presented as non-hemolytic, but O136 as F4 and F5 positive; – isolates of O159 are established from 11th to 20th day as non-hemolytic, F5 +; – strains of O157 persist from 11th to 45th day, as always hemolytic and F4 +, and possibly F18 +; – isolates from O148 are detected from day 21 to day 30, such as hemolytic and F4 +; and from O74 only after weaning, from 31st to 45th day, as non-hemolytic, but both F5 + and F4 +. From 11 to 20 day old suckling pigs and from weaned pigs aged 31 to 45 days, we isolated single, non-hemolytic *E. coli* cultures, where we failed to identify the O-serogroups but all were F5 + (Table 1 and Table 2).

Table 1. Characteristics of *E. coli* strains, isolated from difference ages groups of suckling and weaned pigs in semi-industrial pig farm

Category and age of the pigs in days	**„O“** serologic group	Presence of hemolysin (+/–)	Adhesive factors (F)	Production of enterotoxins	Mechanism of action
Suckling pigs 1– 10 days	O8	–	F5	STA	EPEC
	O20	–	F6	STA	EPEC
	O136	–	F5, F4	LT, STA, STB	EPEC, ETEC
Suckling pigs 11– 20 days	O8	–	F5	STA	EPEC
	O20	–	F6	STA	EPEC
	O136	–	F5, F4	LT, STA, STB	EPEC, ETEC
	O157	+	F4	LT, STB, STA	ETEC, EHEC
	O159	–	F5	STA	EPEC
	ONT	–	F5	STA	EPEC
Suckling pigs 21– 30 days	O8	+	F4	LT, STB, STA	ETEC
	O20	–	F6	STA	EPEC
	O136	–	F4	LT, STB, STA	ETEC
	O148	+	F4	LT, STB, STA STB,	ETEC
	O157	+	F4, F18?	STA, SLT	ETEC, EHEC, Edema Disease
Weaned pigs 31–45 days	O8	+	F5, F4	LT, STB, STA	EPEC, ETEC
	O74	–	F5, F4	STA	EPEC
	O157	+	F4, F18?	STB, STA, SLT	ETEC, EHEC Edema Disease
	ONT	–	F5	STA	EPEC

Table 2. Spectrum and dynamics of intestinal *E. coli* strains from different „O”- serologic groups in suckling and weaned pigs in semi-industrial pig farm

„O” serologic group	Age in days			
	1 – 10	11 – 20	21 – 30	31 – 45
O 8	■	■	■	■
O 20	■	■	■	
O 136	■	■	■	
O 159		■		
O 157		■	■	■
O 148			■	
O 74				■

■ – detected „O” serologic group

Isolated from suckling pigs from the 1st to 20th day of birth, *E. coli* strains were predominantly non-hemolytic (O8, O20, O136, O159 and ONT), predominantly positive for F5 and F6, producing STA and displayed as EPEC. Only two hemolytic isolates were typed as O157:F4+, producing LT, STB and STA and exhibiting ETEC and EHEC. Isolates of *E.coli* from pigs before weaning at 21-30 days of age and after weaning from 31st to 45th day of birth were predominantly hemolytic from serogroups O8, O148 and O157, F4+, and O157 possibly also F18+, producing LT, STA, STB and SLT, occurring as ETEC, EHEC and causing edema disease. In addition, from both groups of pigs, we also isolated and non-hemolytic colibacteria, which were typified as O20:F6, O136:F5 and F6, O74:F5 and F4 and ONT, positive for F5 (Table 1).

The results of testing one part of the isolated *E. coli* strains to 17 antimicrobials from different pharmacological groups are presented in Table 3. It is clear from this that the highest degree of sensitivity was registered to: amikacin – 100%, kanamycin and SMS+trimethoprim – 90% and to gentamicin, enrofloxacin and ciprofloxacin – 80%. To florfenicol, apramycin, cefuroxime and flumequine were sensitive from 50 to 75% of the isolates. To 5 of the remaining 7 AMM, the strains showed a high degree of resistance to: – oxytetracycline and spectinomycin – 90%, amoxicillin – 85% and to tilmicosin and thiamulin – 70%. Significant resistance, from 30 to 40%, was also found to lincomycin-neomycin, cefuroxime and colistin. To 8 of the means were not found resistant strains.

Table 3. Sensitivity of *E. coli* strains, isolated from suckling and weaned pigs to antimicrobial means

№	Antimicrobial means	Strains / num.	Sensitive	Interm. sensitive	Resistance
1	Amoxicillin	20	0	15	85
2	Streptomycin	20	10	0	90
3	Gentamicin	20	80	20	0
4	Kanamyci	10	90	10	0
5	Amikacin	20	100	0	0
6	Apramycin	10	70	30	0
7	Cefuroxime	10	50	10	40
8	Oxytetracycline	10	0	10	90
9	Tilmicosin	20	10	20	70
10	Linco+Neomycine	10	40	20	40
11	Florfenicol	20	75	10	15
12	Colistin	20	40	30	30
13	Flumequine	10	50	50	0
14	Enrofloxacin	10	80	20	0
15	Ciprofloxacin	10	80	20	0
16	Tiamulin	10	0	30	70
17	SMS+Trimethoprim	10	90	10	0

Discussion

The fitting of the breeding flock with gilts pigs from different farms and countries is a factor of particular importance for the epizootiological status of the same and for the clinical condition of the pigs. The fact that in the suckling pigs and weaned pigs in pig farm are recorded manifestations of colibacteriosis in pigs is in accordance with the data of Dimitrova (2009), Dragoycheva et al. (2011), Petkova et al. (2014) on the distribution of the disease in the industrial pigs. It is also confirmed that in one farm can be persisted strains of several O-serogroups, both hemolytic and non-hemolytic, for what reported Dragoycheva et al. (2011) and Dimitrova et al. (2014a, 2014b). Interest is represented, some serogroups (074, 0136, O148 and 0159) which have so far been rarely registered or established for the first time, all the more, so for representatives of 0136 there is evidence, that for the humans are enteroinvasive *E. coli* (EIEC).

The obtained results give us reason to accept the position established by Francis (2002) that *E. coli* strains isolated from pigs up to 10 days old are from different non-hemolytic „O“ groups, F5, F6 and F4 positive, produced predominantly STA toxin, and by mechanism of pathogenic action defined as EPEC and less often as ETEC.

The group of suckling pigs aged 11 to 20 days is presented as a transient, because they are predominantly non-hemolytic strains, F5 and F6 positive, producing only the STA toxin and only in single cases and hemolytic strains of O157:F4, producing LT , STB and STA toxins as they are found by Dimitrova et al. (2014a, b). The results of the study of samples from the following age groups clearly show that both the colienteritis and the edema disease are caused predominantly by haemolytic strains of *E. coli*, in the case of O8:F4, O148:F4 and O157:F4 and possibly F18, producing LT, STA, STB, and SLT. Interesting is the fact that the strains of O8 are already presented as hemolytic and F4+, which means they have a dual character, as suggested by Dimitrova et al. (2014b) and Petkova (2017). Unlike Fairbrother et al. (1994) and Francis (2002), who assume that weaned pigs are colonized by hemolytic ETEC only, we have isolated from pigs before and after weaning and non-hemolytic strains positive for both F5 and F6 and for F4, which presents them as EPEC and ETEC. This is attributable to intensive production, the presence of a wide range of different serotypes, and the possibilities of transferring genes responsible for some pathogenic factors between them, as a cases reported by Luppi et al. (2015)

Unlike Fairbrother et al. (1994); Francis (2002) and Lyutskanov & Urumova (2010), Luppi et al. (2015) we isolated *E. coli* strains from 0157 both weaned pigs and suckling pigs from 11 to 30 days of age. Confirmation is given by Dimitrova (2009), Dragoycheva et al. (2011) and Petkova (2017) that the strains of O157 are the first among the cause of enteritis in pigs from the 21st to 30th day of the suckling period and of enteritis and edematous disease after weaning. These results are in unison with the data from the studies of Dragoycheva et al., (2011) that in the etiology of intestinal coliinfection in pigs, strains of EPEC, ETEC and EHEC from different O-serogroups, of which O8, O20 and O157 are confirm, and O74, O136, O148 and O159 appear to be new to the herd, which is explained by imports of gilts pigs.

The fact that isolated *E. coli* strains have the highest susceptibility to amikacin and kanamycin can be explained in addition to their group characteristics (Lyutskanov & Uru-

mova, 2010; Popova, 2013; Yordanov, 2014), and that these means are use less often in veterinary medicine (Petkova, 2017). Sensitivity to other antimicrobial means of aminoglycoside, fluoroquinolone and amfphenicol groups is consistent with their pharmacological characteristics.

Comparing the susceptibility data of the *E. coli* strains to some of the more commonly used and more effective antimicrobials than those found by Dimitrova et al. (2014a, 2014b) and Petkova (2017) for the country in previous periods, it is clear that it has increased to many of them, such as: – gentamicin from 67.5 to 80%; kanamycin from 73.7 to 90%; – apramycin from 58.1 to 70%; – amikacin from 94.1 to 100%; florfenicol from 67.4 to 75%; – flumequine from 29.6 to 50%; – enrofloxacin from 72.7 to 80% and SMS + trimethoprim from 67.9 to 90%, which is assumed to be due firstly to the replacement of the breeding herd and hence to the pathogenic *E. coli* strains and on the other to increased control of selection and use of AMM. The data from resistance tests show that of the 17 antimicrobials tested, this was found in 9 (53%) of them. including. to: – oxytetracycline and streptomycin 90%, – amoxicillin 85%, – tilmicosin and tiamulin 70%. From the analysis it is clear that resistance is characteristic to amoxicillin, cefuroxime and tiamulin and to some degree for oxytetracycline, tilmicosin and lincomycin, but for spectinomycin (90%), linco+neomycin (40%) and colistin (30%) is indicator for the acquired resistance.

Conclusions

In a semi-industrial pig farm where pigs are imported from Western Europe, from suckling and weaned pigs are isolated 37 strains of *E. coli* from 7 O-serogroups, of which hemolytic O8, O148 and O157 and non-hemolytic O8, O20, O74, O136, O159 and ONT. Three of the groups (O8, O20 and O157) were "old", persisting for more than 5 years and the other 4 groups (O74, O136, O148 and O159) were "new", most probably imported with gilts pigs during the last 3 years.

The *E. coli* strains, isolated from suckling pigs of age from 1 to 20 days of birth, are predominantly non-hemolytic (O8, O20, O136, O159 and ONT), positive for F5 and F6, producing STA and showing like EPEC. Only two hemolytic isolates obtained in the second ten days are typified as O157:F4+, producing LT, STB and STA and occurring as ETEC and EHEC.

Isolated from suckling pigs of 21-30 day old and weaned pigs of 31-45 day old *E. coli* strains are predominantly from hemolytic serogroups (O8:F5+ and F4+; O148:F4+ and O157:F4+ and possibly F18+) producing LT , STB, STA and SLT, and are determined by a mechanism of action such as ETEC, EHEC and causing edema disease. In association with them have been identified non-hemolytic strains, such as O20, O136, O74 and some ONTs positive for F6, F5 and F4, predominantly producing STA toxin and occurring as EPEC and less commonly as ETEC.

High sensitivity (50 to 100%) of isolated *E. coli* strains to AMM from the aminoglycosides, fluorinated quinolones and amphenicols groups were found which is responsible to their pharmacological characteristics. The data for susceptibility of *E. coli* strains to some of the more commonly used antimicrobials, in compared to the ones established for the country in previous periods, clearly show that in some of them it is increased. Resistance to 9 (53%) of the tested 17 AMM was found, including over 50% to: – oxytetracyclin and streptomycin (90%), – amoxicillin (85%), – tilmicosin and thiamulin (70%).

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