Bulgarian Journal of Veterinary Medicine (2001), 4, No 1, 43 - 52

COMPARATIVE STUDY ON THE RESISTANCE OF ENTEROTOXICOGENIC *ESCHERICHIA COLI* (ETEC) ISOLATED FROM PIGS IN THE EARLY 80-TIES AND THE LATE 90-TIES

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Summary

Lyutskanov, M., 2001. Comparative study on the resistance of enterotoxicogenic *Escherichia coli* (ETEC) isolated from pigs in the early 80-ties and the late 90-ties. *Bulg. J. Vet. Med*., **4**, No 1, 43 – 52.

A comparative study upon the resistance of enterotoxicogenic *Escherichia coli* (ETEC) isolated in the early 80-ties and the late 90-ties from newborn pigs with clinical signs of colienterotoxicosis was performed. The behaviour to seven of the most commonly used antibiotics from miscellaneous chemical groups was tested.

The tests were performed using the disk diffusion method. The analysis of data was based on the three-stage categorization according to Bauer-Kirby as well as on a comparative study on cumulative curves of resistant isolates.

An significant increase in the percentages of ETEC isolates, resistant to aminoglycosides-aminocyclitoles, tetracyclines and chloramphenicol, as well as to flumequine was observed. The sensitivity to enrofloxacin was preserved.

Key words: cumulative curves, enterotoxicogenic *E. coli*, resistance

INTRODUCTION

The intensive pig breeding often creates prerequisites for the occurrence of a large number of infectious diseases. A particular part of them is paid on enteric colibacillosis, that is almost permanently present in industrial pig-breeding farms (Taylor, 1989; Bertschinger et al., 1996; Gyles, 1996).

Enteric colibacillosis in newborn and growing pigs is etiologically related to some *Escherichia coli* pathovars (Sussman, 1985, 1997; Gyles, 1996). In Bulgaria, its most frequent form in newborn pigs is the enterotoxic one (colienterotoxicosis). The *E. coli* strains, involved in the etiopathogenesis of enteric colibacillosis are those, producing thermolabile (LT) and thermostable (ST) enterotoxins and possessing fimbrial adhesins from the types K-88 (F4), 987P (F6) and less commonly, K-99 (F5) (Bijulsma et al., 1982). The disease is causing serious economical losses resulting from the loss of offsprings, expenditure of medications, bioproducts, labour, time etc. (Sussman, 1985; Soderlind et al., 1988; Nagy et al., 1990).

The measures for disease control consist primarily in chemoprophylaxis and chemotherapy using various antibacterial drugs, influencing the Gram-negative enterobacteria including ETEC as well (Taylor, 1989). The administration of drugs is however not always preceded by testing of the *in vivo* sensitivity of isolates. Thus, such an extensive and unproper use results in the selection of strains with chromosome- or more oftenly, with a plasmid-

determinated resistance to one or more antibacterial drugs. This resistance is one of the greatest challenges to veterinarians in pig-breeding industry (Courvalin and Carlier, 1981; Chaslus et al., 1987; Mathew et al., 1988).

Those facts motivated our comparative study upon the resistance of enterotoxicogenic *E. coli* strains, isolated in the early 80-ties and the late 90-ties. The aim was to follow out the tendencies in the behaviour of ETEC isolates to most commonly used antibiotics and synthetic chemotherapeutics in order to contribute to the elucidation of the problem of bacterial resistance and the need for a differentiated approach in antibiotics' use in every farm, region and the whole industry.

MATERIALS AND METHODS

The study was performed on 39 field *E. coli* strains, isolated in the 80-ties from the Regional Veterinary Station in Burgas and in the Department of Veterinary Microbiology to the Faculty of Veterinary Medicine in Stara Zagora. They were determined to belong to the enterotoxicogenic pathovar via evidencing the presence of the fimbrial antigens K-88, K-99 and 987P by the slide-agglutination test with specific sera (CEVA). For comparison, another 49 *E. coli* isolates from the same pathovar, obtained in the Department of Veterinary Microbiology during the period 1995-1999 were studied. The isolates were *in vitro* tested to 7 chemotherapeutic drugs, most commonly used in the prophylaxis and treatment of porcine colibacteriosis.

The disk diffusion method in a Muller-Hinton agar was used with antibiotic disks containing the following concentrations (μg/disk): streptomycin (National Centre of Infectious and Parasitic Diseases - NCIPD, Sofia, Bulgaria) – 10; gentamicin (NCIPD) – 10; spectinomycin (Sanofi) – 100; tetracycline (NCIPD) –30; chloramphenicol (NCIPD) – 30; flumequine (Sanofi) – 3; enrofloxacin (Bayer) – 5.

The results were interpreted by the three-degree system of Bauer-Kirby according to the updated criteria of NCCLSM (Performance Standards for Antimicrobial Disk Susceptibility Tests, 1997). The percentages of resistant (R) and inermediate (I) isolates were interpreted together (R+I).

The statistical analysis of data was carried out by the method of Fisher's exact criterion. The confidence limits were calculated. For comparison of the resistance towards each antibiotic for the 80-ties and the 90-ties, the exact criterion of Fisher was applied according to the equation:

$$p = \frac{(a+b).(c+d).(a+c).(b+d)}{n.a.b.c.d}$$

where:

a – number of resistant isolates (R+I) for 80-ties; b - number of sensitive isolates (S) for 80-ties; c - number of resistant isolates (R+I) for 90-ties; d - number of sensitive isolates (S) for 90-ties.

The difference was considered significant at a $p<0.01$ level.

Furthermore, the cumulative percentages of the inhibition zones were calculated and used for drawing cumulative curves for trend analysis of each antibiotic for both compared periods. The cumulative curves were drawn by connecting points corresponding of cumulative percentages (Y axis) and the diameter of inhibition zones (X axis).

RESULTS AND DISCUSSION

Tables 1 and 2 present the number and percentages of isolates from the early 80-ties and late 90-ties divided into sensitive

Table 1. Number and percentages of sensitive (S), intermediate (I) and resistant (R) *E. coli* strains, isolated in the early 80-ties (n=39)

Antibacterial agents	Number			Percentage			R+I, %	Confidence limits
	S	I	R	S	I	R		
Streptomycin	17	1	21	43.6	2.6	56.4	56.4	40.4 – 71.7
Gentamicin	33	0	6	84.6	0	15.4	15.4	5.8 – 28.6
Spectinomycin	39	0	0	100.0	0	0	0	0
Chloramphenicol	7	1	1	94.8	2.6	5.2	5.2	0.5 – 14.6
Tetracycline	11	1	27	28.2	2.6	71.8	71.8	56.5 – 84.9
Flumequine	36	3	0	92.3	7.7	7.7	7.7	1.5 – 18.3
Enrofloxacin	39	0	0	100.0	0	0	0	0

Table 2. Number and percentages of sensitive (S), intermediate (I) and resistant (R) *E. coli* strains, isolated in the late 90-ties (n=49)

Antibacterial agents	Number			Percentage			R+I, %	Confidence Limits
	S	I	R	S	I	R		
Streptomycin	17	7	25	34.7	14.3	51.0	65.3	52.7 – 75.9
Gentamicin	26	2	21	53.1	4.1	42.8	46.9	33.0 – 61.1
Spectinomycin	37	7	5	75.5	14.3	10.2	24.5	13.5 – 37.7
Chloramphenicol	33	7	9	67.3	14.3	18.4	32.7	20.2 – 46.6
Tetracycline	10	6	33	20.5	12.2	67.3	79.5	67.0 – 89.7
Flumequine	39	4	6	79.6	8.2	12.2	20.4	10.3 – 32.9
Enrofloxacin	47	1	1	95.8	2.1	2.1	4.2	0.4 – 11.7

(S), intermediate (I) and resistant (R).

It is evident that there were differences in the resistance towards the aminoglycosides – streptomycin and gentamicin for both periods. Thus, the percentage of *E. coli* isolates, resistant and intermediate (R+I) to streptomycin was 56.4% in the beginning of the 80-ties and increased to 65.3% at the end of the 90-ties ($p>0.01$). The differences by respect to gentamicin were higher – 24.5% and 46.9% for both periods respectively ($p>0.01$).

The increasing tendency towards resistance to those two antibiotics is illustrated on Fig. 1 and 2. It could be explained by the wide application of aminoglycosides in the prophylaxis and treatment of diseases in swine and therefore with the onset of resistance as a function of aminoglycoside-modifying enzymes. It must be emphasized on the presence of a detention of the tendency towards increase in the resistance to streptomycin (Fig.1). This is however valid for values corresponding to inhibition zones under 14 mm. The fact could be explained by the relatively prolonged avoidance of therapeutical and prophylactic use of streptomycin in veterinary practice. This restriction is the result of the introduction of new antibiotics and especially of long-acting preparations during the 90-ties.

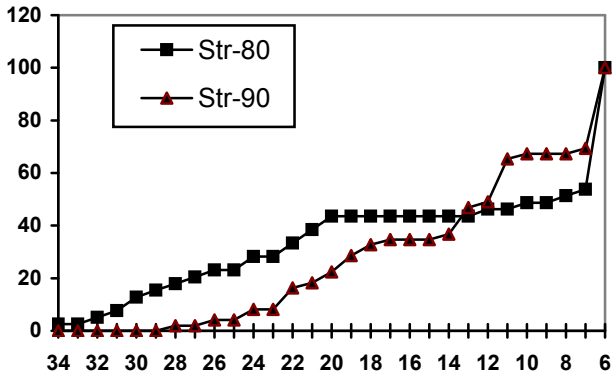

Fig. 1. Cumulative curves of the resistance of enterotoxicogenic *E. coli* against streptomycin in the early 80-ties (Str-80) and in the period 1995-1999 (Str-90). X-axis: diameter of inhibition zones; Y-axis: percentage of resistance.

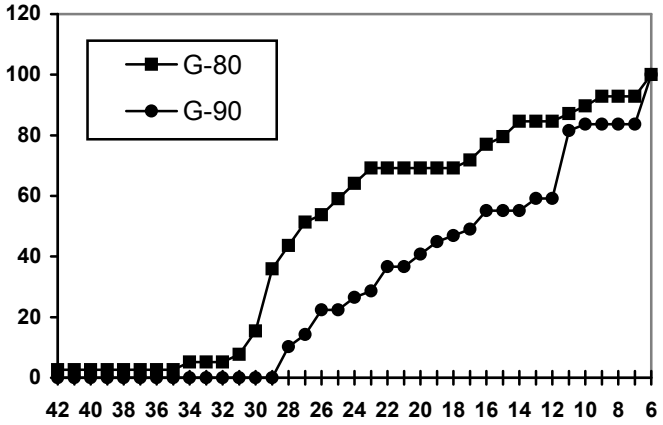

Fig. 2. Cumulative curves of the resistance of enterotoxicogenic *E. coli* against gentamicin in the early 80-ties (G-80) and in the period 1995-1999 (G-90). X-axis: diameter of inhibition zones; Y-axis: percentage of resistance.

Our data for spectinomycin, an antibiotic with a mechanism of action close to that of aminoglycosides and used primarily for veterinary purposes, showed a similar dynamics. All enterotoxicogenic *E. coli* strains, isolated in the early 80-ties were *in vitro* sensitive. Fifteen years later, the percentage of resistant strains from the same pathovar was 10.2% and together with the intermediate organisms (14.3%), the spectinomycin-resistant strains consisted 24.5% of all tested isolates ($p>0.01$). Those differences are illustrated by the cumulative curves on Fig. 3.

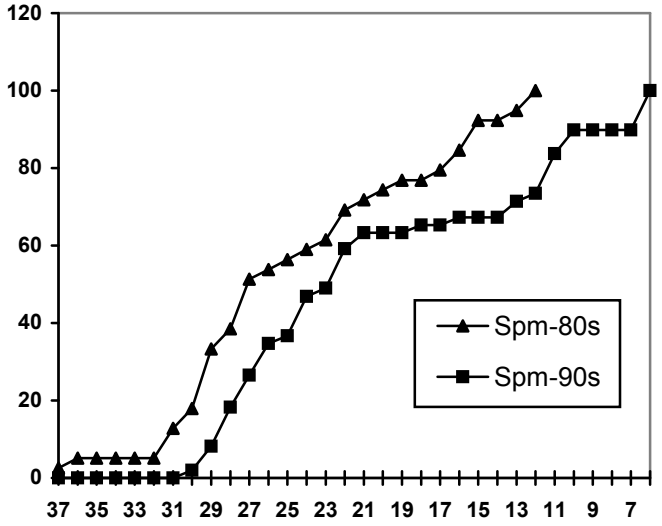

Fig. 3. Cumulative curves of the resistance of enterotoxicogenic *E. coli* against spectinomycin in the early 80-ties (Spm-80) and in the period 1995-1999 (Spm-90). X-axis: diameter of inhibition zones; Y-axis: percentage of resistance.

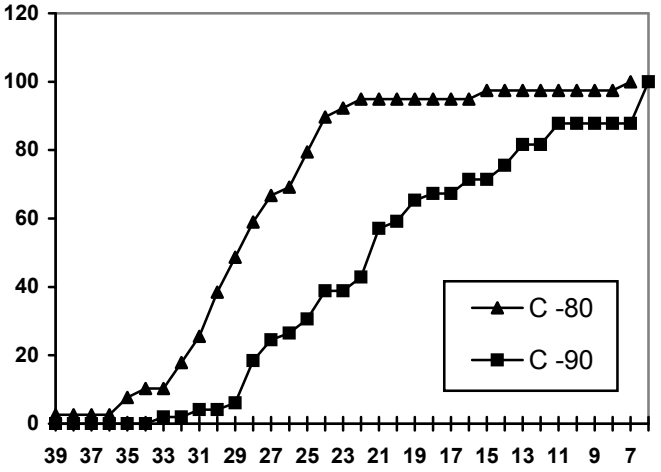

Fig. 4. Cumulative curves of the resistance of enterotoxicogenic *E. coli* against chloramphenicol in the early 80-ties (C-80) and in the period 1995-1999 (C-90). X-axis: diameter of inhibition zones; Y-axis: percentage of resistance.

With respect to chloramphenicol, the percentage of resistant ETEC in the early 80-ties was very low (2.6%) taking into account the 20-year history of its applica-

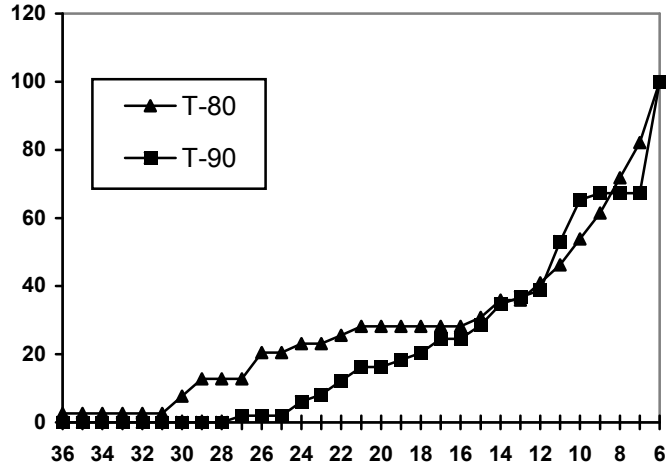

Fig. 5. Cumulative curves of the resistance of enterotoxicogenic *E. coli* against tetracycline in the early 80-ties (T-80) and in the period 1995-1999(T-90). X-axis: diameter of inhibition zones; Y-axis: percentage of resistance.

tion. The percentage of intermediate strains was similar – 5.2%. Within the period 1995-1999, the resistant isolates (R+I) amounted to 13.2%, the difference being statistically insignificant ($p>0.01$).

The change in ETEC sensitivity is due, in our opinion, to the frequent use of chloramphenicol withiout precise indications for both prophylactic and therapeutical purposes. It was included for a long time into the prophylactic programmes of the industrial stock-breeding farms, here including the large pig breeding farms. This use was motivated by the sharp increase in the incidence of salmonelloses in growing and fattening pigs during that period, requiring the application of an antibiotic with a good effect against *Salmonella spp.* such as chloramphenicol. This extensive selective load resulted in augmentation of resistant strains not only among salmonellae isolates but among other enterobacteriae including the enterotoxicogenic *E. coli*. The differences in the percentage of resistant isolates are confirmed by the respective cumulative curves (Fig. 4). For now, chloramphenicol is excluded from clinical use in food producing animals because of the risk of aplastic anaemia.

Data from studies upon ETEC resistance to the tetracycline group showed that as early as during 1981-1986, the percent of sensitive strains was 28.2%. By the end of 90-ties, this percent decreased to 20.5% ($p>0.01$) with 79.5% resistant and intermediate isolates. This result corresponds to the data of other authors (Langlois et al., 1988; Mathew et al., 1998) that reported a low percentage of sensitivity of toxin-producing *E. coli* pathovars to teracyclines. The explanation of this fact could be found with the easier development of resistance based on permeability barriers overcome and moreover, with the extensive selective load connected with the unproper use of tetracyclines in pig forages as feed additives. That is reason for the right shift of tetracycline cumulative curves and their closer

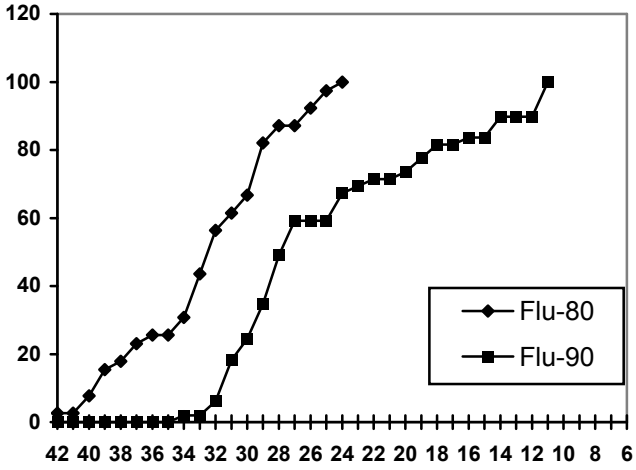

Fig. 6. Cumulative curves of the resistance of enterotoxicogenic *E. coli* against flumequine in the early 80-ties (Flu-80) and in the period 1995-1999 (Flu-90). X-axis: diameter of inhibition zones; Y-axis: percentage of resistance.

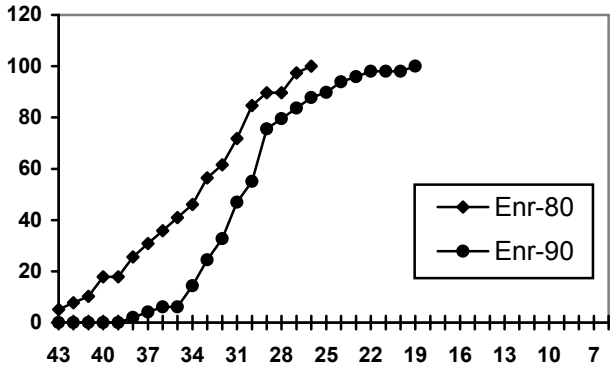

Fig. 7. Cumulative curves of the resistance of enterotoxicogenic *E. coli* against enrofloxacin (Baytril) in the early 80-ties (Enr-80) and in the period 1995-1999 (Enr-90). X-axis: diameter of inhibition zones; Y-axis: percentage of resistance.

location to the X-axis (Fig. 5).

The resistance to both tested fluoroquinolones – flumequine and enrofloxacin (Baytril) was rarely encountered in the beginning of 80-ties before the wide introduction of those preparations in the practice. This is primarily due to their mechanism of action – suppresion of the

bacterial enzyme DNA-gyrase (topoisomerase) that is responsible for the supercoiling of DNA in bacterial genome. Thus, the resistance to fluoroquinolones is chromozome-determined and is realized primarily via mutation.

At the end of the studied period, when the use of fluoroquinolones became routine, the percentage of flumequine–resistant isolates became 12.2% ($p>0.01$) - an evidence that ETEC isolated from pigs have overcome the genetic mechanisms of sensitivity to this chemotherapeutic. According to Everett et al. (1996), this change is realized via the mechanisms of active efflux resulting in loss of extramembrane porins or by mutations in *gyr*-genes. The factor, favourizing the increased resistance could be the wide use of flumequine in the middle 90-ties because of its broad spectrum and acceptable price. It could be supposed that this is the reason for the bigger distance between cumulative curves although they are located predominantly in the sensitive area (Fig. 6).

Towards enrofloxacin, at the end of the studied period, only single resistant or intermediate isolates were found out. This could be seen on cumulative curves, located entirely in the left part of the coordinate system (Fig. 6). Our data suggest that enrofloxacin have to be used as a strategic reserve when there is a need for therapeutical control of enteric colibacillosis in newborn piglets.

The analysis of results showed that for the majority of tested antibiotics and synthetic chemotherapeutics, used in the prophylaxis and therapy of porcine enteric colibacillosis, there was a clear tendency towards increase in the number of resistant isolates. This implies a revision of the approach and the schedule in the treatment of this infectious disease. The treatment, especially on a population level, must be preceeded by a precise etiologic diagnosis and an *in vitro* test of strains' sensitivity to a number of suitable antibacterial drugs.

REFERENCES

Bertschinger, H. U., J. M. Fairbrother, N. O. Nielsen and J. F. Pohlenz, 1996. *Escherichia coli* infection: In: Diseases of Swine, Iowa State University Press, 487 - 488.

Bertschinger, H.U., 1996. Enteric colibacillosis. In: Diseases of Swine, Iowa State Uniwersity Press, 489-497.

Bijulsma, L. G. W., De Nijus, A., Van der Meer, C. J. and F. Frik, 1982. Different pig phenotypes affect adherrence of *Escherichia coli* to jejunial bruch borders by K88 ab, K88 ac or K88 ad antigen. *Infection and immunity*, **37**, 89 -894.

Chaslus-Dancla, E., G. Gerbaud, J. L. Martel, M. Lagorce, J. P. Lafont and P. Courvalin, 1987. Detection of a second mechanism of resistance to gentamicin in animal strains of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*, **31**, No 8, 1274-1277.

Courvalin, P. and C. Carlier, 1981. Resistance towards aminoglicoside-aminocyclitotol antibiotics in bacteria. *Antimicrobial Agents and Chemotherapy*, **1**, No 8 (Suppl.A), 57-69.

Everett, M. J., Y. E. Jin, V. Ricci and L. J. V. Piddock, 1996. Contribution of individual mechanisms fluorquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. *Antimicrobial Agents and Chemotherapy*, **4**, No 10, 2380-2386.

Gyles, C. L., 1996. *Escherichia coli* in domestic animals and humans. CAB International Wallingform, Oxon, Ox10 8DE, UK.

Langlois, B. E., K. A. Dawson, I. Leak and D. K. Aaron, 1988. Antimicrobial resistance of fecal coliforms from pigs in a herd not exposed to antibacterial agents from 126 months. *Journal of Veterinary Microbiology*, **18**, 147-153.

Mathew, A. G., W. G. Upchurch and S. E. Chattin, 1998. Incidence of antibiotic resistance in fecal *Escherichia coli* isolated from commersial swine farms. *Journal of Animal Science*, **76**, 429-434

Nagy, B., T. A. Casey and H. W. Moon, 1990. Phenotype and genotype of *Escherichia coli* isolated from pigs with post-weanind diarrhoea in Hungary. *Journal of Clinical Mictobiology*, **28**, 651-653.

Perfomance Standards for Antimicrobial Disk Susceptibility Tests, Sixth Edition, 1997, NCCLS, M2-A6, **17,** No 1.

Soderlind, O., B. Thafvelin and R. Mollby, 1988. Virulence factors in *Escherichia coli* strains isolated from Swedish pigs with diarrhoea. *Journal of Clinical Mictobiology*, **26**, 879-884.

Sussman, M., 1985. The Virulence of *Escherichia coli*: Reviews and Methods. Academics Press, London, 143-156.

Sussman, M., 1997. *Escherichia coli* - Mechanisms of Virulence. Cambridge University Press, The Edinburg Building, Cambridge, 6B2 2RU, UK, 33-39.

Taylor, D. I., 1989. Colibacteriosis. In: Pig Diseases. The Burlington Press (Cambridge) Ltd., Foxon, Cambridge, 44-49.

Paper received 22.11.1999; accepted for publication 22.03.2000

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