In vitro susceptibility of *Escherichia coli* strains isolated from diarrhoeic lambs and goat kids to 14 antimicrobial agents

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The *in vitro* activities of 14 anti-microbial agents were determined against 92 strains of *E. coli* isolated from lambs (60 strains) and kids (32 strains) affected by neonatal diarrhoea. The overall percentage of resistant strains to streptomycin, sulphadimethoxine and tetracycline was very high (above 70%). A high level of resistance (from 30% to 50%) to ampicillin, kanamycin, neomycin and chloramphenicol was also detected. The *E. coli* strains were highly susceptible to cephalosporins, polymyxin and quinolones. Most of the strains showed multiresistance: 77.2% of isolates were resistant to at least two antibiotics, 55.4% were resistant to at least four antibiotics and 33.7% were resistant to at least six antibiotics. A total of 34 antibiotypes could be distinguished.

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INTRODUCTION

Neonatal diarrhoea is one of the most common and economically devastating problems encountered in farm animals (Holland, 1990). Certain *E. coli* strains are an important cause of diarrhoea in all farm animal species (Holland, 1990; Wray *et al.*, 1993). The role of enterotoxigenic *E. coli* (ETEC), which produces enterotoxins and expresses fimbrial colonization factors in neonatal diarrhoea, has been well established. In addition, other non-enterotoxigenic *E. coli* strains that produce other toxins [verotoxigenic *E. coli* (VTEC) and necrotoxigenic *E. coli* (NTEC)] or cause a characteristic histological lesion [attaching and effacing *E. coli* (AEEC)] have also been associated with neonatal diarrhoea in domestic animals (Holland, 1990; De Rycke, 1991). Although the etiological diagnosis in small ruminants is not determined for a large percentage of cases of neonatal diarrhoea, application of anti-microbial therapy is a common feature in clinical practice. However, high proportions of resistant *E. coli* strains in farm animals and even in animal feed lots have been reported (Coates & Hoopes, 1980; Jackson, 1981; Wood *et al.*, 1983; Prescott *et al.*, 1984; Gonzalez & Blanco, 1985, 1986, 1989; Aalbæk *et al.*, 1991; Adesiyun, 1992; Wray *et al.*, 1993; Blanco *et al.*,1993). The aim of this study was to evaluate the susceptibility of *E. coli* strains isolated from diarrhoeic lambs and kids to different antimicrobial agents.

MATERIALS AND METHODS

E. coli strains

The study was performed with 92 strains of *E. coli* isolated from lambs (60 strains) and goat kids (32 strains) affected by neonatal diarrhoea. The bacterial strains were isolated in our laboratory between 1989 and 1992 from 51 outbreaks of neonatal diarrhoea on farms located in the central region of Spain. One strain per animal was selected. None of the strains studied produced F5 or F41 fimbrial antigens or heat-stable enterotoxin STa (Cid *et al.*, 1993). Fifty-four of the strains were selected because they produce one of the following potential virulence factors: 21 strains produced F17 fimbrial antigen, 16 were VTEC, 12 were NTEC and five produced heat-labile enterotoxin. The remaining 38 strains, which did not produce any of the fimbrial antigens or toxins studied, were selected for comparison.

Faecal samples from all animals from which *E. coli* strains were selected were negative to rotavirus and coronavirus as assessed using a comercial ELISA kit (Tetravalent, Vétoquinol, Magny-Vernois, France).

Antimicrobial agents

The anti-microbial agents studied were provided by the manufacturers: ampicillin (Gema, Barcelona, Spain), cephalexin (Antibióticos, Madrid, Spain), cefazolin (Antibióticos), cefamandole (Lilly, Alcobendas, Madrid, Spain), polymyxin B (Sigma Química, Alcobendas, Madrid, Spain), streptomycin (Antibióticos), kanamycin (Rasfler, Madrid, Spain), gentamicin (Indukern, Barcelona, Spain), neomycin (Indukern), nalidixic acid (Casens-Fisons, Zaragoza, Spain), oxolinic acid (Indukern), sulphadimethoxine (Indukern), chloramphenicol (Fluka Chemika-Biochemika, Alcobendas, Madrid, Spain), tetracycline (Indukern). The antibiotics were dissolved and diluted as recommended by the manufacturers. Fresh dilutions of all compounds were prepared daily.

Anti-microbial susceptibility testing

In vitro susceptibility tests were performed by the agar dilution method according to the recommendation of the National Committee for Clinical Laboratory Standards (NCCLS, 1990). Mueller–Hinton agar (Difco) was used for the agar dilution procedure. The standarized inocula were prepared by inoculating each strain into 2 mL of brain heart infusion (BHI) broth (Difco). The tubes were incubated at 37°C until the turbidity reached a density corresponding to McFarland nephelometer standard no. 0.5. Bacterial cultures were diluted 1:10 in 0.9% NaCl solution and 1 μL of each bacterial suspension was applied by use of a Steers replicator to the anti-microbial-containing agar plates, yielding an inoculum of approximately 10^4 CFU per spot. Plates were incubated at 37°C for 24 h. Minimal inhibitory concentration (MIC) was read as the lowest concentration of anti-microbial agent that suppressed visible bacterial growth. One colony or a slight haze in which no distinct colonies could be observed was ignored. Reference strain *E. coli* ATCC 25922 was included as an internal control in all parts of the study. The range of interpretative categories of susceptibility for each anti-microbial agent were those recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1990).

Statistical analysis

The significance of differences was determined by chi-square (χ^2) test. A level of $P < 0.05$ was considered to be significant.

RESULTS

The results of *in vitro* susceptibilities to 14 anti-microbial agents of the 92 strains of *E. Coli* studied are shown in Table 1. The range and MIC_{50} and MIC_{90} (the lowest concentration of anti-microbial agent at which there was no growth of 50% and 90% of strains) of each of the 14 agents, as well as the percentage of *E. coli* resistant strains were determined (Table 1).

The overall percentage of resistant strains to streptomycin, sulphadimethoxine and tetracycline was very high (above 70%). Moreover, a high level of resistance (from 30% to 50%) to ampicillin, kanamycin, neomycin and chloramphenicol was also detected. *E. coli* strains were highly susceptible to cephalosporins (only one ovine isolate was resistant to cefamandole and one caprine isolate was resistant to cephalexin), polymyxin and the two quinolone compounds tested (only one ovine isolate was resistant to nalidixic acid).

In general, the different groups of anti-microbial agents tested showed similar activities against the *E. coli* strains isolated from lambs and from kids. However, significantly higher rates of resistance ($P < 0.05$) were found in kids' isolates than in those from lambs to ampicillin (59.3% in strains from kids vs. 35% in strains from lambs), kanamycin (65.5% vs. 35%), gentamicin (28.1% vs. 5%), neomycin (53.1% vs. 18%) and sulphadimethoxine (90.6% vs. 66.6%). Higher MIC_{50} values were observed for caprine isolates than for ovine isolates for ampicillin (64 μg/mL vs. 4 μg/mL), streptomycin (128 μg/mL vs. 64 μg/mL), kanamycin (256 μg/mL vs. 4 μg/mL), neomycin (64 μg/mL vs. 1 μg/mL) and chloramphenicol (64 μg/mL vs. 8 μg/mL).

No significant differences between the 54 strains producing a potential virulence factor and the 38 non-fimbriated non-toxigenic strains were found in the rates of resistance against any of the anti-microbials tested. However, VTEC strains were significantly less resistant to kanamycin and neomycin than $F17^+$ strains, and significantly less resistant to streptomycin, kanamycin and neomycin than non-fimbriated non-toxigenic strains.

Fourteen strains (15.2%) were sensitive to all the anti-microbials tested. Only seven strains (7.6%) were resistant to

Table 1. *In vitro* susceptibilities of 92 *E. coli* strains isolated from diarrhoeic lambs and kids to 14 anti-microbial agents

Anti-microbial agent*	*MIC*†			Resistant strains No.(%)
	Range	50%	90%	
Ampicillin (≥32)	≤0.062–≥512	8	256	40 (43.5)‡
Cephalexin (≥32)	0.125–128	4	8	1 (1.1)
Cefazolin (≥32)	0.5–16	1	2	0 (0)
Cefamandole (≥32)	0.125–64	2	8	1 (1.1)
Polymyxin B	≤0.062–8	8	8	0 (0)
Streptomycin (≥64)	1–≥512	64	≥512	65 (70.6)
Kanamycin (≥64)	0.5–≥512	8	≥512	42 (45.6)‡
Gentamicin (≥16)	0.125–64	1	16	12 (13)‡
Neomycin (≥64)	0.25–256	2	64	28 (30.4)‡
Nalidixic acid (≥32)	1–64	2	8	1 (1.1)
Oxolinic acid (≥32)	0.125–1	0.25	0.5	0 (0)
Sulphadimethoxine (≥512)	2–≥512	≥512	≥512	69 (75)‡
Chloramphenicol (≥32)	2–≥512	8	≥512	43 (46.7)
Tetracycline (≥16)	1–≥512	128	256	67 (72.8)

†Minimal inhibitory concentrations (*MICs*) of all agents are given in μg/mL. 50% and 90%, *MICs* for 50% and 90% of the strains tested respectively. *Numbers in parentheses are *MIC* interpretative standards (μg/mL) of resistant category according to NCCLS recommendations. ‡Significantly higher rates of resistance were found in kids' isolates than in those from lambs.

one antibiotic. Thus, most of the strains showed multiresistance: 77.2% of isolates were resistant to at least two antibiotics, 55.4% were resistant to at least four antibiotics and 33.7% were resistant to at least six antibiotics. About 16% of the strains were resistant to seven or eight antibiotics.

VTEC strains showed the lowest rate of multidrug resistance with an average of 2.1 resistances per strain, whereas $F17^+$, NTEC, LT^+ and non-fimbriated non-toxigenic strains showed rates of 4.6, 4, 4 and 4.2 respectively.

A total of 34 antibiotypes could be distinguished. The most frequently occurring are those shown in Table 2.

Table 2. The most frequent antibiotic resistance patterns of the *E. coli* strains studied

Resistance patterns	Resistant strains	
	No.	(%)
AM,S,K,N,SD,C,TE	9	(9.8)
AM,S,SD,C,TE	8	(8.7)
S,K,N,SD,TE	7	(7.6)
AM,S,K,SD,C,TE	6	(6.5)
S,SD,TE	6	(6.5)
S,SD	3	(3.3)
SD	3	(3.3)

AM, ampicillin; S, steptomycin; K, kanamycin; N, neomycin; SD, sulphadimethoxine; C, chloramphenicol; TE, tetracycline.

DISCUSSION

Studies of anti-microbial resistance of intestinal *E. coli* from different animal species showed an increased incidence of resistance over the years as a result of the widespread use of anti-microbial drugs in animals (Jakson, 1981; Coates & Hoopes, 1980; Smith & Lovell, 1981; Hanzawa *et al.*, 1984; Prescott *et al.*, 1984; Aalbæk *et al.*, 1991; Blanco *et al.*, 1993; Wray *et al.*, 1993). The use of drugs does not induce resistance but rather provides an intense selection pressure which eliminates the susceptible bacteria in the host and spares the resistant ones (Hinton *et al.*, 1986). This is particularly true for bacteria that inhabit the intestinal tract because they are readily exposed to the actions of antibiotics administered orally either at prophylactic or therapeutic levels or when incorporated in animal feed as growth promoters (Hinton *et al.*, 1986). Studies of *E. coli* isolated from different animal species showed a relationship between the degree of anti-microbial drug use and extent of resistance (Linton, 1977). Therefore, antibiotic resistance is usually prevalent in intensively reared calves, pigs and poultry, whereas adult grazing cattle and sheep have a very low incidence of resistance among their faecal *E. coli* (Linton, 1977; Hinton *et al.*, 1985).

Most of the veterinary reports on resistance of *E. coli* relate to calves, pigs and poultry. Comparatively few reports on anti-microbial resistance of ovine and caprine *E. coli* strains are available. In general, the frequency of resistance to different anti-microbial classes used in this study was higher than those reported in other studies. Wray *et al.* (1993) reported that more than 50% of ovine *E. coli* strains were resistant to chlortetracycline, sulphonamides and ampicillin. The incidence of resistance to other drugs tested by these authors was lower: neomycin (38%), furazolidone (< 1%), chloramphenicol (18%), apramycin (< 1%) and streptomycin (48%), and 30.2% of the isolates were sensitive to all the anti-microbials tested. Moreover, the percentage of sensitive *E. coli* strains isolated from newborn lambs by Corlu *et al.* (1990) to different anti-microbials was higher than those described in this study. On the other hand, the percentages of resistant strains from lambs and kids to streptomycin, tetracycline, kanamycin and neomycin reported by Adesiyun (1992) in Trinidad are similar to those found in this study. However, the incidence of resistance to ampicillin and chloramphenicol found by Adesiyun (1992) was very low in comparison with that found in this study.

The percentages of resistant strains to ampicillin, aminoglycosides, sulphonamides, tetracycline and chloramphenicol found in this study are similar to those previously reported for bovine *E. coli* (Coates & Hoopes, 1980; Sherwood *et al.*, 1983; Gonzalez & Blanco, 1989; Blanco *et al.*, 1993; Wray *et al.*, 1993). However, higher rates of resistance to nalidixic acid and cephalothin (a first generation cephalosporin) have been described for bovine *E. coli* isolates (Pohl *et al.*, 1991; Blanco *et al.*, 1993) than those observed in this study.

Some reports suggest that pathogenic *E. coli* are more likely to be resistant than non-pathogenic strains (Gyles *et al.*, 1977). However, there is no conclusive evidence for this suggestion, since in several studies ETEC strains have been found to be more sensitive to antibiotics than non-ETEC strains (Brunton *et al.*, 1980; De Boy *et al.*, 1980; Gonzalez & Blanco, 1985). Furthermore, no major differences were observed between the incidence of resistance recorded for pathogenic and non-pathogenic *E. coli* isolates from pigs (Franklin, 1984). On the other hand, Blanco *et al.* (1993) found that *E. coli* strains isolated from diarrhoeic calves were more resistant to antibiotics and had a higher rate of multidrug resistance than those isolated from healthy calves. A similar finding has been reported for chickens (Chularisi & Suthienkul, 1989). Nevertheless, Adesiyun (1992) found that the prevalence of resistance to antibiotics was similar for strains isolated from diarrhoeic and non-diarrhoeic lambs and kids.

The strains included in this study were all isolated from animals with diarrhoea, although their association with the disease could not be established. Thus, the classification of $F17^+$, VTEC and NTEC strains as potentially pathogenic is tentative, since there is little information about the role of this fimbriae and toxins in disease of sheep and goats (Wray *et al.*, 1993).

The results of this study for VTEC are in contrast to those previously described for strains isolated from diarrhoeic calves by Gonzalez and Blanco (1989), who found that VTEC were significantly more resistant to different antibiotics than NTEC strains and non-VTEC non-NTEC strains, and that multiple drug resistance was higher in VTEC than in NTEC and non-VTEC non-NTEC strains. Burnens *et al.* (1995), however, found that 41 of

50 VTEC strains isolated from cattle in herds with and without calf diarrhoea were sensitive to all the antibiotics tested.

The reason for the significantly high rates of resistance of kids' isolates for different anti-microbials is not clear, since the housing conditions, the management of animals and the use of antibiotics in sheep and goat farms in the central region of Spain are broadly similar. Nevertheless, Adesiyun (1992) also found higher rates of resistance in kid than in lamb isolates for streptomycin, tetracycline, kanamycin and neomycin.

In this study the highest rates of resistance have been detected against the anti-microbial drugs most commonly used by Spanish veterinary clinics for treatment of diarrhoea in farm animals (sulphonamides, tetracyclynes, streptomycin), while the *E. coli* strains studied were highly susceptible to rarely used anti-microbial agents (cephalosporins, quinolones, polymixins). Neonatal diarrhoea in lambs and kids is often treated with anti-microbial drugs. However, antibiotic therapy is frequently ineffective, partly due to the presence of drug-resistant strains and the failure to identify drug sensitivity. The therapeutic implications of our findings in the treatment of neonatal diarrhoea in small ruminants should be emphasized.

In this study a very high rate of multi-resistant *E. coli* strains was found. More than 30% of the strains were resistant to at least six anti-microbial agents. Multiple drug-resistant isolates of *E. coli* strains from farm animals have been reported worldwide (Coates & Hoopes, 1980; Jakson, 1981; Prescott *et al.*, 1984; Gonzalez & Blanco, 1985, 1986, 1989; Aalbæk *et al.*, 1991; Adesiyun, 1992; Wray *et al.*, 1993; Blanco *et al.*, 1993). The seriousness of the problem arises from the fact that once multiresistant organisms develop they can persist in the host or in the environment in the absence of antibiotic selection (Hinton, 1986). This may be of particular importance since cattle (Orskov *et al.*, 1987; Borzyck *et al.*, 1987; Gonzalez & Blanco, 1989) and probably sheep and goats (Doyle & Schoeni, 1987; Beutin *et al.*, 1993) might act as reservoirs of certain *E. coli* strains pathogenic for man. Nevertheless, as suggested by Hinton (1986), the importance of animals as a source of resistant *E. coli* should not be taken out of context because the medical use of antibiotics is principally responsible for the presence of resistance among bacterial isolates from man.

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