J. Dairy Sci. 86:3927–3932

In Vitro Antimicrobial Susceptibility of *Escherichia coli* Isolates from Clinical Bovine Mastitis in Finland and Israel

T. Lehtolainen,* A. Shwimmer,† N. Y. Shpigel,†
T. Honkanen-Buzalski,‡ and S. Pyörälä*
*University of Helsinki
Faculty of Veterinary Medicine
FIN-04920 Saarentaus, Finland
†Hebrew University of Jerusalem
Koret School of Veterinary Medicine
Rehovot 76100, Israel
‡National Veterinary and Food Research Institute
FIN-00580 Helsinki, Finland

ABSTRACT

Minimal inhibition concentration (MIC) values of 100 Finnish and 100 Israeli *Escherichia coli* isolated from clinical bovine mastitis were determined for ampicillin, cephalexin, ceftazidime, dihydrostreptomycin, gentamicin, tetracycline, trimethoprim-sulfadiazine, and ciprofloxacin by an agar dilution method. The in vitro antimicrobial susceptibility of the *E. coli* isolates was high; only 27% showed resistance to one or more tested antimicrobial agents. Fifteen percent of the Israeli isolates and 14% of the Finnish isolates were resistant to tetracycline, 3 and 16% to cephalexin, 10 and 7% to ampicillin, 13 and 9% to dihydrostreptomycin, and 4 and 2% to trimethoprim-sulfadiazine. No gentamicin-, ceftazidime-, or ciprofloxacin-resistant isolates were detected. Eleven percent of all the isolates were resistant to two or more antimicrobial agents. Tetracycline was most often associated with multiresistant patterns. Most of the multiresistant isolates had very high MIC values, whereas most of those that were resistant to only one tested antibiotic had MIC values close to the susceptibility breakpoint. Antimicrobial resistance appeared to pose no problem in *E. coli* isolated from mastitic milk of both countries. This is probably due to the controlled use of antimicrobial agents in the treatment of dairy herds. Some differences were present in the resistance patterns, which may reflect the different use of antimicrobial agents in these two countries.
(Key words: antimicrobials, *Escherichia coli*, mastitis, susceptibility)

Received April 14, 2003.
Accepted June 25, 2003.
Corresponding author: T. Lehtolainen; e-mail: tanja.lehtolainen@helsinki.fi.

Abbreviation key: DHS = dihydrostreptomycin, **MIC** = minimal inhibition concentration, $\mathbf{MIC_{50}}$ = minimal inhibition concentration for 50% of isolates tested, $\mathbf{MIC_{90}}$ = minimal inhibition concentration for 90% of isolates tested, **TS** = trimethoprim-sulfadiazine.

INTRODUCTION

Environmental bovine mastitis caused by coliform bacteria has increased in many herds and countries (Lam, 1996). The great majority of these coliform bacteria belong to *Escherichia coli*. *Escherichia coli* originate from the cow's environment and infect the udder via the teat canal (Eberhart, 1979). The proportion of coliform mastitis in clinical mastitis varies between countries. In Finland, less than 20% of clinical mastitis is caused by coliforms (Pyörälä and Honkanen-Buzalski, 1994), whereas in Israel more than 60% (Shpigel et al., 1998) of clinical mastitis is caused by coliforms. This is probably the result of differences in environmental factors and herd management. In Finland, small herds are mostly kept indoors and confined to stanchion barns. In Israel, large herds with very high yielding cows are kept in loose housing systems in hot weather.

Broad-spectrum antimicrobial agents are generally used to treat coliform mastitis (Andersson, 1989; Erskine et al., 1991), although there is no convincing evidence that antimicrobials are an effective (Erskine et al., 1992; Pyörälä et al., 1994; Pyörälä and Pyörälä, 1998; Erskine, 2000). Antibiotic therapy may be useful if the host response is compromised, as during the puerperal period, or if the growth of bacteria in the milk is abundant (Erskine et al., 2002; Rantala, 2002). However, the use of antimicrobial agents causes selection pressure toward antimicrobial resistance among bacteria (Aarestrup, 1999; Prescott, 2000), and resistance to one antimicrobial agent can be linked with resistance

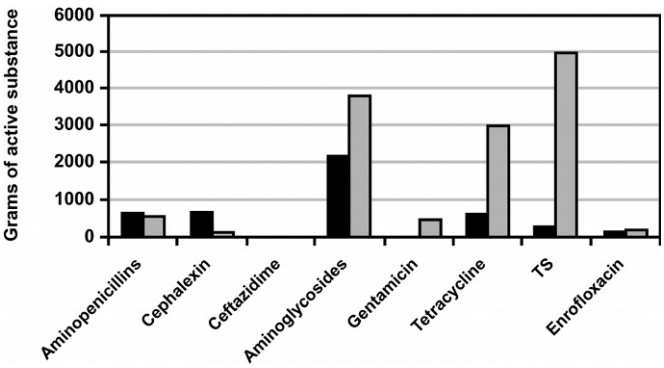

Figure 1. Amounts of aminopenicillins, cephalexin, ceftazidime, aminoglycosides, gentamicin, tetracycline, trimethoprim-sulfadiazine (TS), and enrofloxacin used for dairy cows in Finland[1] (solid bars) and in Israel[2] (gray bars). The data is shown as grams of active antimicrobial per thousand cows. [1]Data from Finish Ministry of Agriculture, estimated from statistics from the National Agency of Medicines. [2]Data from Hacklait, the Israeli wholesaler.

against other antimicrobials (Levy et al., 1976; Prescott, 2000). The resistance patterns of the bacterial populations can vary between countries or even herds, which may reflect the quantitative and qualitative aspects of antimicrobial treatment (Aarestrup, 1999; DANMAP, 2001; Österbland et al., 2001).

The use of antimicrobial substances in animal therapy has been recorded in Finland since 1994 (Figure 1). β-Lactam antibiotics are the most commonly used antimicrobial agents for cattle (Helin et al., 2000). Mastitis is the main indication for the use of antimicrobial agents in dairy cows (Honkanen-Buzalski and Huovinen, 1999). Most mastitis cases are caused by gram-positive bacteria and have mainly been treated with systemically administered penicillin, or sometimes with intramammarily administered aminoglycosides combined with β-lactam antimicrobials or oxytetracycline. Acute clinical mastitis caused by gram-negative bacteria is often treated systemically with trimethoprim-sulphonamide or enrofloxacin.

In Israel, no systematic surveillance of antimicrobial administration for animals exists. However, all medicine for cattle is supplied by the wholesaler, who keeps annual records of consumption and the types of medicine used in dairy herds (Figure 1). The main indications for antimicrobial treatments in dairy herds are metritis, and mastitis caused by coliform bacteria. Antimicrobials used are mainly tetracycline, gentamicin, and a trimethoprim-sulfonamide combination. Penicillin combined with dihydrostreptomycin (**DHS**) is also used parenterally for foot problems and intramammarily for dry cow treatment.

The incidence of *E. coli* mastitis, herd management, and the use of antimicrobial agents differ between Finland and Israel. Consequently, the aim of this study was to determine the in vitro antimicrobial susceptibility of *E. coli* bacteria isolated from clinical mastitis in Finland and Israel, and to identify possible differences in susceptibility of the isolates between these two countries.

MATERIALS AND METHODS

Bacterial Isolates

A total of 200 *E. coli* isolates from acute clinical cases of bovine mastitis were studied: 100 from Finland and 100 from Israel. The isolates were collected from 90 dairy farms in Finland and from 27 farms in Israel. A milk sample was aseptically taken from the affected quarter of the cow before any antimicrobial treatment and cultured using standard methods. Isolates were stored in a semi-solid broth (Lab Lemco broth, Oxoid, Hampshire, UK) at room temperature. They were presumptively identified as *E. coli* by phenotypic methods, including colony morphology on blood agar, Gram stain, and growth on EMB agar, and identification was later confirmed using the API 20E test (bioMérieux, Marcy l'Etoile, France) at the National Veterinary and Food Research Institute, Kuopio, Finland.

Escherichia coli ATCC 25922, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 29213 (NCCLS, 1999) were used as reference strains. Both test isolates and reference strains were prepared in suspension at a concentration of 10^7 cfu/ml, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1999).

Antimicrobial Agents

Animal inhibition concentration (**MIC**) values of the isolates were analyzed for eight different antimicrobials: ampicillin (ampicillin sodium salt, Sigma Chemical Co., St. Louis, MO, lot 127H0285), cephalexin (cephalexin hydrate, Sigma, lot 24H0420), ceftazidime (ceftazidime pentahydrate, Sigma, lot 35H0473), DHS (dihydrostreptomycin sesquisulfate salt, Sigma, lot 66H04142), gentamicin (gentamicin sulfate, Sigma, lot 27H0744), tetracycline (tetracycline, Sigma, lot 38H1324), trimethoprim-sulfadiazine (**TS**) (trimethoprim, Sigma, lot 105H0136; sulfadiatsin, Sigma, lot 96H1175), and ciprofloxacin (ciprofloxacin*HCl, Bayer Ag, Levekusen, Germany, lot 303477A) by an agar dilution method (NCCLS, 1999). The antimicrobial stock solutions were prepared and diluted as recommended by their respective manufacturers to a concentration of 1280 μg/ml. The TS was made up to a ratio of 1:5. The actual concentrations for both compounds in the stock solution were 213.33 μg/ml for trimethoprim and 1066.66 μg/ml for sulfadiazine.

The stock solutions of each antibiotic were further diluted and mixed with molten Mueller-Hinton agar. The final concentrations ranged in two-step dilutions from 128 μg/ml to 0.5 μg/ml for ampicillin, cephalexin, DHS, tetracycline, and TS; from 64 μg/ml to 0.25 μg/ml for gentamicin; and from 16 μg/ml to 0.0625 μg/ml for ciprofloxacin and ceftazidime, which also had a concentration of 32 μg/ml. These antimicrobials are all options or group representatives of potential subtances for the treatment of coliform mastitis. Ciprofloxacin was chosen to represent enrofloxacin since enrofloxacin is extensively metabolized to ciprofloxacin in cattle (Kaartinen et al., 1995).

Antimicrobial Susceptibility Testing

All isolates were tested at the National Veterinary and Food Research Institute, Helsinki, Finland. Antimicrobial agars were inoculated with the respective bacterial suspensions using the Automatic Multipoint Inoculator (Mast Diagnostics Ltd., Merseyside, U.K.) with 3-mm metal pins. The volume of the suspension drop was 2 μl, and it contained 2×10^4 cfu of *E. coli* (NCCLS, 1999). Growth of the inoculates was determined after incubation at 37°C for 20 h by observing the agar plates directly from above. The MIC value was recorded as the lowest concentration of the antibiotic to inhibit growth of the inoculated bacteria (NCCLS, 1999).

The MIC breakpoint values used for in vitro susceptibility were 8 μg/ml for ampicillin, cephalexin, DHS, and TS; 4 μg/ml for gentamicin and tetracycline; 0.25 μg/ml for ciprofloxacin; and 2 μg/ml for ceftazidime (NCCLS, 1999). The breakpoint values for DHS, ciprofloxacin, and ceftazidime were set at the level of streptomycin, enrofloxacin, and ceftiofur, respectively, given in the NCCLS document. An isolate was considered resistant to a certain antimicrobial if the MIC value were higher than the MIC breakpoint value for susceptibility of that antimicrobial. Values for MIC for 50 and 90% of isolates tested (**MIC_{90}** and **MIC_{50}**) were calculated for all antimicrobials tested.

Statistical Analysis

Differences in the frequencies of resistance to antimicrobials among the isolates were determined by Pearson's chi-squared test. A value of $P < 0.05$ was considered to be significant.

RESULTS

The MIC values of *E. coli* isolates from both Finland and Israel were similar and relatively low (Table 1). The majority of the isolates were susceptible to the antimicrobial agents studied. A total of 30% of Finnish and 24% of Israeli isolates were resistant to one or more antimicrobials. Of the Finnish isolates, 16% were resistant to cephalexin, compared with only 3% of Israeli isolates; however, only one Finnish isolate had a high MIC value (>128 μg/ml); other isolates classified as resistant, according to the NCCLS breakpoint, were at the borderline. For the other antimicrobial agents, the proportion of resistant strains was higher for Israeli isolates than for Finnish ones. Ten percent of Israeli isolates and 7% of Finnish isolates were resistant to ampicillin, 13 and 9% to DHS, 15 and 14% to tetracycline and 4 and 2% to TS, respectively. No gentamicin-, ciprofloxacin-, and ceftazidime-resistant isolates were detected. The difference in the number of resistant isolates between the two countries was statistically significant ($P < 0.01$) for cephalexin only.

In total, 32 isolates (16%), 20 from Finland and 12 from Israel, were resistant to only one antimicrobial, namely cephalexin, tetracycline, DHS, or ampicillin (Table 2). Twenty-two isolates (11%), 10 Finnish and 12 Israeli, were resistant to two or more antibiotics in 10 different combinations. From the total amount of resistant isolates, 41% were multiresistant. Tetracycline was included in all but one multiresistant pattern; DHS and ampicillin were also often present. Most of the multiresistant isolates had very high MIC values, whereas most of those that were resistant to only one tested antimicrobial had MIC values close to the susceptibility breakpoint.

Whereas MIC_{50} values for Finnish and Israeli isolates did not differ markedly, differences were present between MIC_{90} values (Table 1). For cephalexin, the Finnish isolates had one dilution higher MIC_{90} value (16 μg/ml) than Israeli isolates (8 μg/ml), whereas for ceftazidime and TS, the MIC_{90} values were one dilution higher among Israeli (0.5 and 4 μg/ml, respectively) vs. Finnish isolates (0.25 and 2 μg/ml, respectively). For DHS and tetracycline, the Israeli isolates had clearly higher MIC_{90} values (32 and 64 μg/ml, respectively) than the Finnish isolates (8 μg/ml for both).

DISCUSSION

The in vitro antimicrobial susceptibility of the examined *E. coli* isolates from bovine mastitis was high compared with results from previous studies (Bishop et al., 1980; Sogaard, 1982; Anderson, 1989; Trolldenier, 1995). However, because some of these studies have used different susceptibility testing methods, comparisons should be made with caution. Only 27% of our isolates were resistant to one or more antimicrobial agents. The most common resistance was to tetracy-

Table 1. Cumulative percentages of MIC values of the *Escherichia coli* isolates from bovine mastitis from Finland (n = 100) and Israel (n = 100). The MIC susceptibility breakpoint value of each antimicrobial is indicated in bold.[1]

MIC (μg/ml)	<0.0625	0.063	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128
Ampicillin														
Finnish					0	2	27	83	**93**	93	93	93	94	100
Israeli					0	5	24	73	**90**	91	91	91	94	100
Cephalexin														
Finnish					0	0	0	3	**84**	99	99	99	99	100
Israeli					0	0	1	36	**97**	100	100	100	100	100
Ceftazidime														
Finnish	31	32	89	100	100	100	**100**	100	100	100	100			
Israeli	2	2	43	89	99	100	**100**	100	100	100	100			
DHS[2]														
Finnish				0	0	0	15	77	**91**	92	92	92	96	100
Israeli				1	1	13	82	87	**87**	89	90	94	96	100
Gentamicin														
Finnish			2	4	43	95	97	**100**	100	100	100	100		
Israeli			0	3	72	99	100	**100**	100	100	100	100		
Tetracycline														
Finnish					0	2	34	**86**	92	92	92	94	99	100
Israeli					0	4	72	**85**	86	86	86	92	98	100
TS[3]														
Finnish				56	60	86	97	98	**98**	98	98	98	99	100
Israeli				1	6	19	83	93	**96**	96	96	96	96	100
Ciprofloxacin														
Finnish	100	100	100	**100**	100	100	100	100	100	100				
Israeli	99	99	100	**100**	100	100	100	100	100	100				

[1]MIC = minimal inhibition concentration.

[2]DHS = dihydrostreptomycin.

[3]TS = trimethoprim-sulfadiazine.

cline, accounting for 15% of all isolates. This is a relatively low figure as compared with other published studies, where the proportion of resistant isolates has ranged from 26 to 74% (Sogaard, 1982; Anderson, 1989; Trolldenier, 1995; DANMAP, 2001). We found no isolates resistant to gentamicin, although 9% resistance was reported in an earlier US study (Anderson, 1989). In addition, the proportion of resistant isolates to ampicillin and DHS in our study, 9 and 11%, were clearly lower than the corresponding figures in the previous studies: 18 to 82% (Bishop et al., 1980; Sogaard, 1982; Anderson, 1989) and 58% (Davidson, 1982), respec-

Table 2. Resistance patterns among the *Escherichia coli* strains isolated from bovine mastitis in Finland and in Israel.

Resistance patterns					Number of isolates	
					Finnish	Israeli
Tetracycline	DHS[1]	Ampicillin	Cephalexin	TS[2]	1	0
Tetracycline	DHS	Ampicillin	Cephalexin		1	0
Tetracycline	DHS	Ampicillin	TS		0	4
Tetracycline	DHS	Ampicillin			0	1
Tetracycline	DHS	Cephalexin			1	0
Tetracycline	DHS	TS			1	0
Tetracycline	DHS				1	4
Tetracycline	Ampicillin	Cephalexin			1	0
Tetracycline	Ampicillin				2	1
DHS	Ampicillin				2	2
Tetracycline					6	5
DHS					2	2
Ampicillin					0	2
Cephalexin					12	3
Negative					70	76
Total					100	100

[1]DHS = dihydrostreptomycin.

[2]TS = trimethoprim-sulfadiazine.

tively. Among our resistant isolates, 41% were multiresistant, a slightly lower figure than that found in a previous report, where 50% of the resistant isolates were multiresistant (Sogaard, 1982).

Some studies have shown that antibiotic usage has directly contributed to an increased prevalence of resistance (Aarestrup, 1999; DANMAP, 2001). The use of antimicrobial agents in dairy herds is well controlled in both Finland and Israel. Antimicrobials are used in cattle for therapeutic purposes only; subtherapeutic or growth-promoting use is prohibited. The total amounts of antimicrobial agents consumed in dairy herds have remained relatively low compared with some other areas (Figure 1) (DANMAP, 2001; Gorbach, 2001). This could be one reason for the low level of resistance found among *E. coli* bacteria isolated from bovine mastitis compared with other studies.

Coliform mastitis is most often caused by fecal flora originating from the same or another cow (Linton et al., 1984). Coliform bacteria isolated from mastitis may thus reflect the general resistance situation in the herd and can be considered more as an indicator of bacteria than of specific pathogens of the udder. All antimicrobial use in the herd may affect the resistance of coliforms. The more resistant strains of *E. coli* are proposed to originate from calves, due to their greater exposure to antibiotics (Linton et al., 1984). However, neither in Finland nor in Israel are calves excessively exposed to antibiotics because of the restriction imposed on subtherapeutic or growth-promoting use. In Israel, calves are housed separately from cows so they do not share the same bacterial reservoir. In Finland, calves are kept with dairy cattle in the same building. The antimicrobial agents traditionally used for treatment of calves have been DHS, oxytetracycline, and the trimethoprim-sulfonamide combination (Honkanen-Buzalski and Huovinen, 1999). These might have affected the resistance of Finnish isolates by increasing the presence of these antimicrobial agents in the cows' environment.

The only statistically significant difference between the susceptibility of Finnish and Israeli isolates was for cephalexin. In Finland, this first-generation cephalosporin is commonly used as a first choice for intramammary treatment of mastitis, and the total amount used is higher than that in Israel (Figure 1). This could explain the higher proportion of resistant isolates and the slightly higher MIC_{50} value compared with Israeli isolates. In both countries, tetracycline, DHS, and ampicillin showed the highest resistance, and they were also the most frequently found antimicrobial agents in the multiresistant patterns. Israeli isolates had a two-dilution higher MIC_{90} value for DHS and a four-dilution higher MIC_{90} value for tetracycline. These antimicrobial agents are used much more often and in greater amounts in Israel than in Finland (Figure 1).

From tetracycline-, DHS- and ampicillin-resistant isolates, 62, 82, and 88%, respectively, were also resistant to some other antimicrobial. Those antimicrobial agents are often found together in multiresistant patterns, possibly indicating transferable resistance (Österbland et al., 2000; Oppegaard et al., 2001). Multiresistant bacteria also tend to maintain their resistance to a particular antimicrobial even when that antimicrobial is absent from the environment if the other antimicrobials to which the resistance is linked are still present (Levy, 1992; Prescott, 2000; Galland et al., 2001). Not only the amount of antimicrobials used, but also the frequency of use, may be relevant for the emergence of resistance (Levy, 1992).

In conclusion, in vitro susceptibility of *E. coli* isolated from mastitis in Finland and Israel was high, which may indicate the low total use of antimicrobial agents in the cows' environment in these countries. Differences in MIC profiles may reflect differences in the use of antimicrobial agents. Susceptibility of *E. coli* isolated from mastitis could be considered to be an indicator of the general susceptibility situation among bacteria of the herd.

ACKNOWLEDGMENTS

We would like to thank Hannu Sipilä for excellent assistance with the laboratory work. This work was supported by the Finnish Academy and by a grant from the Walter Erhrstöm Foundation.

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