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Characterization of expanded-spectrum cephalosporin resistance in *E. coli* isolates associated with bovine calf diarrhoeal disease

Patricia A. Bradford[a]*, Peter J. Petersen[a], Ian M. Fingerman[a]† and David G. White[b]‡

[a]*Wyeth-Ayerst Research, 401 N. Middletown Rd, Pearl River, NY 10965;* [b]*Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo, ND, USA*

Antibiotic resistance among *Escherichia coli* isolates from diarrhoeal disease in cattle was studied. Many of the isolates were multiply resistant to β-lactams, including expanded-spectrum cephalosporins, aminoglycosides, sulphonamides, tetracycline and fluoroquinolones. In many of the isolates, IEF revealed a strong β-lactamase band compatible with over-expression of the AmpC β-lactamase, either alone or in addition to TEM-type enzymes. Several of the isolates also possessed genes encoding virulence factors associated with animal and human diarrhoeal diseases. These results suggest that the use of antibiotics in animals could lead to a reservoir of antibiotic-resistant bacteria that could potentially infect humans.

Introduction

Pathogenic *Escherichia coli* are one of the most important groups of bacteria causing diarrhoea and extraintestinal infections in humans and animals.[1] There have been many recent reports of morbidity and mortality due to outbreaks of disease-causing *E. coli* that have been attributed to foods of bovine origin or other foods cross-contaminated by beef products or cow manure.[2] The occurrence of toxigenic *E. coli* strains in humans and calves with diarrhoea is well documented and cattle have been considered an important reservoir of Shiga-like toxin (STX)-producing *E. coli* strains involved in human disease.[3]

Bovine calf scours is a severe form of diarrhoea that causes more financial loss to cow-calf producers than any other disease-related problem. Scours is a clinical sign of disease that may have many causes, although *E. coli* has been frequently implicated as the primary bacterial cause in calves.[4] Antibiotics are frequently used by animal owners and veterinarians in the treatment and prevention of scours.

In recent years, bacterial resistance to β-lactam antibiotics has risen dramatically in human pathogens. The use of expanded-spectrum cephalosporins in health institutions contributes to the emergence of such resistance. However, there is limited knowledge concerning the incidence and prevalence of extended-spectrum β-lactamases (ESBLs) among pathogenic veterinary strains of *E. coli* incriminated in animal disease. Therefore, a survey was performed to collect data on the occurrence of antibiotic resistance and virulence factors among *E. coli* strains associated with bovine calf scours in the state of North Dakota, USA. This survey, revealed that about 13% of *E. coli* strains implicated in bovine calf scours were resistant to ceftiofur, an expanded-spectrum cephalosporin used in veterinary medicine. This study was performed to characterize the nature of bacterial resistance to expanded-spectrum cephalosporins.

Materials and methods

Bacterial strains

Samples of *E. coli* obtained from individual bovine calf scours cases during 1996 were examined for antimicrobial susceptibility. *E. coli* were selected from primary agar cultures of either bovine faeces from animals with diarrhoea, or intestinal tissue from septicaemic animals taken post mortem. All of the isolates represented cases that had failed antimicrobial therapy. Thirty-two strains that showed varying degrees of resistance to ceftiofur were chosen for further study. The recipients for mating and transformation experiments were *E. coli* strains C600N and DH5α, respectively.

*Corresponding author. Tel: +1-914-732-4396; Fax: +1-914-732-5671; E-mail: bradfop@war.wyeth.com
†Present address: Rutgers University, Piscataway, NJ, USA. ‡Present address: Food and Drug Administration, Center for Veterinary Medicine, Laurel, MD, USA.

Susceptibility testing

Initial bacterial antibiotic susceptibilities were carried out using Kirby–Bauer disc diffusion assays performed by standard procedures.[5] MICs were determined by microdilution using standard procedures.[6] Antibiotics were obtained from their respective manufacturers.

Characterization of β-lactamase

Isoelectric focusing (IEF) was performed by the method of Matthew *et al.*[7] using an LKB Multiphor apparatus with prepared PAGplates, pH 3.5–9.5 or pH 4.0–6.5. (Pharmacia LKB, Piscataway, NJ, USA). The isoelectric point (pI) of each enzyme was determined by activity staining with nitrocefin (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) following IEF.

Nucleic acid techniques

DNA isolation and PCR were performed by standard techniques.[8] Ribotyping was performed using the RiboPrinter system (Qualicon, Wilmington, DE). PCR was used to detect TEM β-lactamase genes as well as genes for virulence factors including Shiga-like toxins I and II (STX-I, STX-II), *E. coli* attaching and effacing factor (EAE), cytotoxic necrotizing factor I (CNF-I), cytotoxic necrotizing factor II (CNF-II), heat labile enterotoxin (LT), K99 adhesion fimbriae (K99), heat stable enterotoxin a (STa) and heat stable enterotoxin b (STb), using specific oligonucleotide primers.

Results

Susceptibility

MICs of β-lactam antibiotics for the clinical isolates are shown in Table I. All of the isolates tested were resistant to ampicillin and had reduced susceptibility to ticarcillin and piperacillin which suggested the presence of a β-lactamase. The addition of a β-lactamase inhibitor to these penicillins restored susceptibility to some, but not all of the isolates. In addition, 27 of the 32 isolates tested showed increased resistance to the expanded-spectrum cephalosporins, aztreonam and cefoxitin. None of the isolates were resistant to imipenem. Susceptibility to non-β-lactam drugs is shown in Table II. All of the isolates were resistant to kanamycin, streptomycin, sulphisoxazole and tetracycline. In addition, many were resistant to chloramphenicol, gentamicin and trimethoprim–sulphamethoxazole. Four isolates were nalidixic acid resistant but only two strains were ciprofloxacin resistant.

β-Lactamase characterization

IEF results (Table I) showed β-lactamases with pIs of 5.4, 5.6 and 9.0 present in varying patterns among the isolates.

Table I. Isoelectric focusing, TEM β-lactamase detection and MICs of β-lactam drugs for veterinary isolates

No. isolates	pI of β-lactamase	TEM PCR	Probable identity of β-lactamases	Range of MIC (mg/L)										
				AMP[a]	AMC	TIC	TIM	PIP	PTZ	XNL	CAZ	CTX	ATM	FOX	IPM
5	5.4	+	TEM-1	>128	8–32	>128	32–128	>128	2–16	0.25–1	0.12–2	⩽0.06–0.5	⩽0.06–1	4	0.12
17	9.0	-	AmpC	128–>128	32–>128	32–>128	16–>128	16–>128	4–64	1–32	8–64	2–16	2–16	32–>128	⩽0.06–1
5	5.4, 9.0	+	TEM-1, AmpC	>128	64–>128	>128	128–>128	>128	16–>128	8–>128	32–>128	8–>128	16–>128	>128	⩽0.06–1
5	5.6, 9.0	+	TEM-2, AmpC	>128	64–>128	>128	64–128	>128	16–64	2–32	16–32	8–16	4–16	16–>128	0.12–1
ATCC 25922	none		none	4	8	4	8	1	2	0.25	0.25	⩽0.06	0.12	4	0.12
ATCC 35128			TEM-1	>128	8	>128	16	>128	2	0.12	⩽0.06	⩽0.06	⩽0.06	2	0.12

[a]AMP, ampicillin; AMC, co-amoxiclav; TIC, ticarcillin; TIM, ticarcillin–clavulanate; PIP, piperacillin; PTZ, piperacillin–tazobactam; XNL, ceftiofur; CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam; FOX, cefoxitin; IPM, imipenem.

Table II. Ribotype and non-β-lactam antibiogram of bovine isolates

No. of strains	Ribotype[a]	Disc diffusion susceptibility								
		CAM[b]	KM	GM	STR	SUL	SXT	TET	NAL	CIP
1	I	S[c]	R	S	R	R	R	R	S	S
2	I	R	R	R	R	R	R	R	S	S
4	I	S	R	R	R	R	R	R	S	S
2	II	S	R	R	R	R	R	R	S	S
1	II	R	R	S	R	R	R	R	S	S
2	III	R	R	S	R	R	S	R	S	S
2	IV	R	R	R	R	R	R	R	S	S
4	V	R	R	S	R	R	R	R	S	S
2	VI	R	R	S	R	R	R	R	S	S
2	VII	R	R	R	R	R	R	R	R	R
4	U	R	R	R	R	R	R	R	S	S
1	U	S	R	S	R	R	S	R	S	S
2	U	S	R	S	R	R	R	R	S	S
2	U	S	R	S	R	R	I	R	R	S
1	U	S	R	R	R	R	R	R	S	S

[a]Roman numerals indicate a ribotype grouping. U indicates strain is unique based upon ribotyping.
[b]CAM, chloramphenicol; KM, kanamycin; GM, gentamicin; STR, streptomycin; SUL, sulphisoxazole; SXT, trimethoprim/ sulfamethoxizole; TET, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin.
[c]Susceptibility determined by disc diffusion, interpretation based upon NCCLS criteria.[5]

The pI 9.0 β-lactamase was a strong and prominent band which appeared rapidly after staining with nitrocefin. Five isolates produced only the pI 5.4 enzyme, 17 produced only the pI 9.0 enzyme and 10 produced the pI 9.0 β-lactamase in combination with either the pI 5.4 or the pI 5.6 β-lactamase. PCR with TEM-specific primers gave positive results, with plasmid DNA isolated from strains expressing a β-lactamase of pI 5.4 or 5.6 (Table I). Pathogenic veterinary strains that produced only the TEM-type enzyme with a pI of 5.4 were all susceptible to extended-spectrum cephalosporins. Increased resistance to the expanded-spectrum cephalosporins, aztreonam and cefoxitin correlated with the presence of the pI 9.0 β-lactamase and was presumed to be the AmpC of *E. coli* expressed at higher than usual levels.

Ribotyping

The ribogroups determined by ribotyping are shown in Table II. Ten of the isolates produced unique ribotype patterns. The remaining strains could be separated into seven distinct ribogroups. Of these, the most common was designated ribotype I and contained seven isolates. Groups II–VII consisted of two to four isolates. There was no correlation between ribotype and susceptibility, β-lactamase type or carriage of virulence factors.

Characterization of virulence factors

Genes for STX-II, CNF-II, LT and STb were not detected among these isolates although eight strains were positive for the STa enterotoxin and K99 fimbriae genes, indicating that they were enterotoxigenic *E. coli* (ETEC). One isolate was positive for STX-I and EAE and one was positive for CNF-1. The remaining isolates were negative for the virulence factors assayed.

Discussion

Ceftiofur, a veterinary expanded-spectrum cephalosporin, is active against a variety of animal pathogens associated with bovine and swine respiratory disease.[9] Although this antimicrobial is not approved for treatment of bovine calf scours, it is often used to treat bacterial diarrhoeal diseases when strains are multiply resistant to other veterinary antimicrobials. In this study, many of the *E. coli* isolates associated with bovine calf scours were found to be multiply resistant to several antibiotic classes including extended-spectrum cephalosporins. This latter resistance was most likely due to the hyperproduction of the chromosomally encoded AmpC β-lactamase, which is found naturally in *E. coli*, but usually not expressed at high levels. Although there is no direct correlation between the occurrence of cephalosporin resistance and the use of β-lactams in veterinary medicine, these results suggest that the use of these antimicrobials may have contributed to the in-vivo selection of *E. coli* strains that hyperproduce AmpC. No ESBLs were found among this population of strains which suggests that these enzymes do not routinely play a role in expanded-spectrum cephalosporin resistance among veterinary isolates of *E. coli*. However, identification of

several TEM-type β-lactamases among these isolates suggests that the emergence of ESBLs in this population of pathogens is possibly only a matter of time. The somewhat surprising finding of so many *E. coli* isolates that overexpress their AmpC constitutes a serious threat to veterinary β-lactam therapy of *E. coli*-related diseases, as well as serving as a potential reservoir for antibiotic-resistant human pathogens.

The ribotyping data indicated that there was not a clonal outbreak of a single multiply resistant strain, but that several strains arose following a number of independent mutational events. Several of the strains possessed virulence factors that are associated with human diarrhoeal disease. These genes are often encoded on plasmids and may possibly be co-transferred with antimicrobial resistance genes.

The results from this study demonstrated that resistance to front line antimicrobials is present among *E. coli* strains incriminated in bovine calf scours. This combination of virulence coupled with multidrug resistance may pose an increasing threat to successful treatment of *E. coli*-related veterinary diseases in the near future. The extensive use (and some misuse) of antimicrobials has led to the loss of efficacy through emergence of bacterial antibiotic resistance.[10] Although only a few of the isolates in this study possessed virulence factors that are associated with human disease, they were resistant to multiple antibiotics. If these strains were to cross over into the human population, therapeutic options would be limited if antibiotic therapy were necessary.

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