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MODIFICATION OF OUTER MEMBRANE PERMEABILITY AND ALTERATION OF LPS IN VETERINARY ENTEROTOXIGENIC *ESCHERICHIA COLI*.

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) is a worrying cause of diarrhoea in calves and the drug multiresistance phenotype concerning various antibiotic families are of concern. Resistance mechanisms associated with envelope changes (porin expression, efflux pump overexpression, lipolysaccahride (LPS) modification) were studied in 14 ETEC isolates selected for their resistance. We performed determinations of (i) antimicrobials Minimal Inhibitory Concentrations with or without the efflux pump inhibitor phenylalanine arginine β-naphthylamide; (ii) colistin and polymyxin MICs with and without EDTA, (iii) intracellular accumulation of chloramphenicol in presence of an energy uncoupler of pump energy, (iv) and immunodetection of porins and evaluation of porin trimers thermostability.

Results indicated that 9 strains presented significant efflux mechanisms overexpression, among them 8 were resistant to colistin and polymyxin B due to a modification of LPS structure as evidenced by EDTA effect and silver staining electrophoresis. The high resistant strains to colistin and polymyxin exhibited identical LPS patterns. Studies of *E. coli* porins indicated that the majority of strains didn't show modification in their amount, however analysis of porin thermostability showed that porin trimers of some resistant strains were relatively heat-labile, suggesting a misassembly of the functional trimer. The multidrug resistance (MDR) phenotypes detected in these selected ETEC corresponded to association of LPS modifications, abordive assembly of porin trimers and active efflux which drastically alter the antibiotic activity currently used to combat enteric infections caused by this pathogen.

- **INTRODUCTION**

In veterinary medicine, infectious diarrhoea of neonatal calves is associated with high mortality and economic concern that requires the use of broad spectrum antimicrobials associations [14, 36, 40]. One of the commonly reported causative enteropathogen is the enterotoxigenic *Escherichia coli* (ETEC) and several reports have revealed that calves are rapidly colonized by multidrug resistant antibiotic (MDR) strains of *E. coli* within days of birth as the consequence of use of antimicrobials in food animal [14, 15, 24, 26, 37]. Among these strains, β-lactam resistance is increasing due to diverse β-lactamases and plasmid-mediated cephalosporin (AmpC type) resistance [8, 10, 24]. Moreover, most of *E. coli* strains isolated in diarrheic calves show multiresistance to structurally-unrelated antimicrobials [2, 6, 14]. Until now colistin and fluoroquinolones are the antimicrobial agents used in this pathology because most of the isolated *E. coli* strains remained susceptible to these molecules [23, 36, 37]. However, in the last decade polymyxin resistance has emerged in animals and human isolates [2, 4, 10, 15, 24, 44, 48]. Recently, it has been shown that in populations of enteropathogenic strains isolated in a context of neonatal calf diarrhoea, 61-65 % were resistant to one or more substances and 28-77 % were multi-resistant [14, 20, 22, 47].

The aim of the present study was to evaluate the involvement of resistance mechanisms associated to outer membrane (OM) permeability which can affect polymyxins, fluoroquinolones and chloramphenicol in a population of multiresistant ETEC isolated from diarrheic dairy calves.

- # MATERIAL AND METHODS

- ## Bacterial strains and growth media

Fourteen ETEC strains, belonging to the *E. coli* strain collection (2006, various departments of France) isolated in faecal samples of dairy neonatal calves showing diarrhoea prior any antibiotic therapy, were selected according to their noticeable resistance against colistin (Table 1). They were previously assayed for their β-lactamases expression by PCR (TEM, SHV, OXA) and sequenced [41]. Strains 42 and 73 harboured TEM1, OXA30 and overexpressed chromosomic AmpC cephalosporinase, due to *ampC* promoter mutation. The susceptible strain 39 was used as control strain during the determination of Minimal Inhibitory Concentrations (MICs) for the various isolates. The strain *E. coli* JM 109 that expresses OmpC and OmpF porins was used as control for protein assays. Bacteria were routinely grown in Luria Bertani (LB) or Muller-Hinton (MH) (BioMérieux) broth, 24h at 37°C.

- ## Antibiotic susceptibility

Susceptibilities (MIC) to five structurally-unrelated classes of bacterial drugs were carried out using a two fold broth microdilution method in MH broth according to the guidelines of the French antibiogram committee of Society for Microbiology to evaluate the resistance phenotype (http://www.sfn.asso.fr/nouv/general.php?pa=2). The results were scored after 18h at 37°C and are expressed as MICs. The MIC was determined as the lowest concentration that inhibited visible growth. Values are medians of at least three independent experiments and are presented in mg/L.

The following antimicrobials were selected: ofloxacin, ciprofloxacin, nalidixic acid, chloramphenicol, gentamicin and polymyxins (colistin, polymyxin B) were purchased from Sigma (Saint Quentin Fallavier, France) and cefpirome was a gift of Sanofi-Aventis (France).

The effect of efflux pumps on susceptibilities were carried out using a fixed concentration (50µM) of the efflux pump inhibitor Phenylalanine Arginine-β-Naphthylamide (PAβN) (Sigma) during determination of quinolones, cefpirome, gentamicin and chloramphenicol activities [18, 28]. Susceptibility to colistin and polymyxin B were determined with two fixed concentrations (0.1 and 0.5 mM) of the cationic chelator ethylenediaminetetraacetate (EDTA) (Sigma) that chelates magnesium present in lipopolysaccharide (LPS) chains and sensitizes Gram-negative bacteria to membranotropic antimicrobials [1]. Values are medians of at least three independent experiments and are presented in mg/L.

- **Outer membrane protein immunoblotting and lipopolysaccharide detection**

Whole bacterial proteins corresponding to standardized cell pellets collected from exponential-phase bacteria in LB broth were solubilized in loading buffer at 96°C. Samples (0.02 optical density at 600 nm) containing OM porins were separated on SDS-PAGE gel and Western immunoblots were routinely prepared by electrotransfer onto nitrocellulose sheets. The sheets were incubated sequentially: (i) with polyclonal antibodies directed against denatured porin OmpC and OmpF, and (ii) with an anti- rabbit IgG antibody conjugated to alkaline phosphatase [12, 30, 42]. About LPS assays, the profile was analysed after electrophoresis on SDS-PAGE gel with tricine buffer and gel was stained by silver staining [44, 46].

- **Chloramphenicol accumulation**

Accumulation of [^{14}C] chloramphenicol in 2×10^{10} colony-forming units/mL was assayed in sodium phosphate buffer (50mM; pH7) supplemented with 5mM $MgCl_2$ according to the method described previously [9]. In order to collapse the membrane energy which is the energy-driven source of the AcrAB efflux pump, 50µM carbonyl cyanide *m*-

chlorophenylhydrazone (CCCP), was added 10 min before the radio-labelled chloramphenicol [9, 31]. Samples were removed at set intervals and were filtered and washed. The filters were dried and radioactivity was measured with a liquid scintillation counter (Perkin-Elmer Life and Analytical Sciences, Wellesley, MA). Values are expressed as counts per minute per unit of optical density [9].

- **RESULTS AND DISCUSSION**

- **Susceptibility to colistin and polymyxin B, and lipopolysaccharide patterns**

Eight out of fourteen strains (17, 20, 30, 40, 48, 50, 75 and 98) exhibited a resistance to both colistin and polymyxin B (Table 1). The MIC values were significantly higher for colistin (64 to 8 µg/mL) than for polymyxin B (16 to 2 µg/mL). The remaining strains, 26, 39, 42, 73, 80 and 96 were susceptible to both antimicrobials with MIC values between 0.5 to 2 µg/mL. Importantly, the susceptibility of resistant strains to both colistin and polymyxin B, was restored totally or partially by EDTA, a chelator of magnesium cation. Whatever the concentration tested, 0.1 and 0.5 mM (data not shown), the MICs were reduced by two to five folds except for strains 39 and 80 (Table 1).

As polymyxins affect the OM structure, analysis of the LPS patterns of the *E. coli* strains was carried out [1, 17, 45]. The Fig. 1 presented the different LPS profiles and their modifications suggest a significant modification of the LPS structure in these resistant strains. Five strains, 26, 30, 39, 80 and 96 were grouped in cluster I containing the polymyxin susceptible strains, with the exception of the strain 30. Strains 40, 48, 50 and 75 were aggregated in cluster II in which 40, 48 and 75 exhibited marked low colistin-polymyxin susceptibility. Strains 42, 73 and 98 belonged to cluster III and the two strains 17 and 20 were different. The protective barrier afforded by OM is mainly due to the LPS structure constituting the outer leaflet of the

membrane. Alterations of LPS structure associated or not with over expression of multidrug efflux pumps contribute to a multiresistant phenotype constitution [11, 16, 32, 35, 50]. In two third isolates studied, the high resistance to polymyxin B and colistin was associated with modifications of LPS altering the activity of both antimicrobials. The decrease in anionic surface charges due to substitution of phosphate groups in LPS accounted for reduced binding sites for the cationic polymyxin B and colistin [49]. Colistin resistance can result from addition of cationic groups to the LPS, loss of LPS production by mutations, deletions or insertion sequence in genes involved in the lipid A biosynthesis [34]. A large panel of genes and operons are involved in qualitative modification of the LPS including genes and operons coding for enzymes that are directly involved in LPS modifications (*pmrC*, *pmrE genes* and the *pmrHFIJKLM* operon) and regulatory genes (*pmrAB, phoPQ* two-component systems and their regulators: *mgrB* gene, *crrAB)* [27, 34, 39]. The recent discovered plasmid-mediated *mcr-1* gene, responsible for horizontal transfer of colistin resistance, encodes the MCR-1 protein, a phosphoethanolamine transferase which can add a phosphoethanolamine to lipid A, generating a more cationic LPS [39]. An involvement of LPS sizes in the permeation of lipophilic drugs have been demonstrated [50]. Finally, few studies have shown that efflux pumps can also contribute to polymyxin resistance [34]. In *E. c*oli isolates that exhibited different LPS patterns, the activity of polymyxin B and colistin was increased by EDTA that weakened the OM and restored the activity of polymyxins [1, 17]. These data are in agreement with previous studies where EDTA is able to destabilize the OM increasing polymyxin and colistin uptake [21, 32, 45]. Moreover, this conferred resistance was stable since no revertant was obtained in a colistin free medium and this suggests the selection of mutations and activation of regulatory systems such as PmrA/PmrB and PhoP/PhoQ by environmental stresses [38, 49].

- **Susceptibility to a range of structurally-unrelated antimicrobials**

The majority of the strains showed high MICs for nalidixic acid, ciprofloxacin, ofloxacin and chloramphenicol (Table 2). Quinolones are able to self-promoted their uptake by diffusion across the LPS barrier [3]. Consequently, the EDTA increased the activity of nalidixic acid on resistant strains and MICs decreased about three fold (data not shown). This fits in with the highly resistance of these strains to colistin and polymyxin B. Moreover, majority of strains presented a high resistance to chloramphenicol while all strains were intermediate or resistant for gentamicin. Interestingly, only three strains 30, 42 and 73 were resistant to cefpirome. To investigate a possible role of efflux mechanism in the resistance level, PAβN, an efflux pump inhibitor, was assayed in combination with antimicrobials [11, 28]. The addition of PAβN induced increased nalidixic acid and chloramphenicol susceptibilities with MICs decreasing to two to three fold [25]. However, PAβN had a weak or no effect on fluoroquinolones, gentamicin and cefpirome activities suggesting that a PAβN-sensitive active efflux is not the major mechanism involved in the resistance for these molecules [33]. In this case, a mutation in the antibiotic target can be hypothesised.

- **Chloramphenicol accumulation**

To evaluate the effect of the efflux pump on the accumulation of chloramphenicol inside the strains, the intracellular accumulation of radio-labeled chloramphenicol was performed. Accumulation was determined for ten isolates in the absence or in the presence of carbonyl cyanide *m*- chlorophenylhydrazone (CCCP), an energy uncoupler that collapses the membrane energy [28, 33]. The steady-state concentration of chloramphenicol was measured after 10 minutes of incubation. A low level of chloramphenicol accumulation was found in the absence of CCCP in the majority of strains studies, compared to the susceptible strain 39 (Fig. 2). Importantly, a significant increase was obtained in presence of CCCP during the

incubation assay, demonstrating the activity of an energy dependent efflux mechanism for chloramphenicol. This noticeable efflux contributes to antibiotic resistance in these isolates by decreasing the intracellular drug concentration (Fig. 2). The ratio of chloramphenicol accumulation (value obtained on plateau with CCCP/value obtained on plateau without CCCP) was particularly high in strains 20, 30, 40, 50, 73 and 75 (Table 3). The results showed a good correlation between the values of MIC and the impact of CCCP on the efflux level of chloramphenicol as observed by Moreira *et al.* [31]. The activities of chloramphenicol and nalidixic acid were increased in presence of PAβN in *E. coli* resistant isolates for which the main efflux pump is AcrAB-TolC (Table 2). In these MDR strains, the increase of chloramphenicol accumulation in the presence of CCCP demonstrated the role of an active efflux process driven by a proton motive force [29, 33, 40, 43].

- **Immunodetection of porins**

The immunodetection of porins with polyclonal antibodies directed against OmpC and OmpF monomers indicated no modifications of their expression in the various strains (Fig. 3). Regarding the porin stability, the trimers produced in strains 40, 48 ,50, 73, 75, 80, 96 and 98 exhibited a relative thermal unstability at 60°C, some monomers are observed in contrast to other porin from control strain *E. coli* JM 109 and other strains (data not shown). This trimer-monomer transition that occurred at relative low temperature, 60°C compare to 75°C, suggested a misfolding of the functional trimer [30, 42].

This trimer unstability may be thus explained by the production of an altered LPS chains that induced an abordive insertion of porins into the OM [5, 7, 13, 42]. In these veterinary ETEC strains, obtained prior any known antibiotherapy, it is worthy of note that the LPS modifications correlate with a polymyxin resistance which could involve the PmrA-PmrB and PhoP-PhoQ two-component systems [34]. Concerning the resistance to β-lactams, it was

confirmed the high prevalence of *E. coli* strains producing extended spectrum AmpC β-lactamases and ESBL in human digestive microbiota [41].

All together, these various mechanisms (modifications of LPS, misassembly of porin trimers and active efflux), conjointly expressed exhibited by the tested ETEC resistant strains, drastically impair the efficiency of antimicrobials currently used to combat diarrhea the most prevalent cause of death in calves [20]. Importantly, the detection of such membrane changes raises questions about the genetic or environmental events that have contributed to the emergence and/or spread of such adapted strains.

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Strains	MIC (µg/mL)	
	Colistin	Polymyxin B
17 17+EDTA*	8 4	4 2
20 20+EDTA	16 2	4 1
26 26+EDTA	2 0.5	1 0.5
30 30+EDTA	16 1	4 0.5
39 39+EDTA	< 2 2	0.5 0.5
40 40+EDTA	64 2	8 1
42 42+EDTA	1 n.d**	n.d n.d
48 48+EDTA	32 8	16 2
50 50+EDTA	8 1	2 0.5
73 73+EDTA	1 n.d	n.d n.d
75 75+EDTA	64 2	8 2
80 80+EDTA	2 2	0.5 0.5
96 96+EDTA	2 1	1 0.5
98 98+EDTA	32 4	4 2

EDTA* at concentration: 0.1mM; n.d**: not determined. The values are the means of the three independent assays.

Table 1: Susceptibilities of enterotoxigenic *Escherichia coli* strains isolated from calves to colistin and polymyxin B in the absence or in the presence of EDTA (used as chelator of divalent cations present in bacterial envelope)

Strains	Nalidixic acid	Ciprofloxacin	Ofloxacin	Cefpirome	Gentamicin	Chloramphenicol
17	> 512	32	16	1	64	256
17+PAβN*	128	16	8	1	128	128
20	> 512	2	4	0.5	> 64	512
20+PAβN	128	1	1	0.5	> 64	128/64
26	> 32	< 0.015	0.03	0.25	4	1
26+PAβN	< 0.125	< 0.015	0.03	0.125	2	0.5
30	> 512	0.5	1	4	4	512
30+PAβN	256	0.5	0.5	>4	4	128
39	0.5	< 0.015	0.06	0.06	4	1
39+PAβN	< 0.125	< 0.015	0.03	0.125	2	0.25
40	> 512	8	16	0.5	> 64	256
40+PAβN	128	8	4	2	> 64	64
42	2	<0.015	0.06	> 8	4	> 256
42+PAβN	1	<0.015	0.03	> 8	2	64
48	> 512	128	64	1	32	128/256
48+PAβN	128	64	16	0.5	32	128
50	> 512	> 8	> 8	0.125	> 32	> 512
50+PAβN	128	> 8	8	0.25	32	128
73	n.d**	32	16	> 4	512	128
73+PAβN	n.d	16	8	> 4	256	n.d
75	> 512	8	8	0.5	4	256
75+PAβN	128	8	4	2	4	64
80	> 32	<0.015	0.06	0.06	4	1
80+PAβN	0.25	0.015	0.03	0.06	1	0.5
96	> 512	64	32	0.25	> 64	4
96+PAβN	128	32	16	0.25	> 64	2
98	> 512	128	64	0.5	64	128
98+PAβN	128	64	16	0.5	> 64	64

PAβN* at concentration 50µM; n.d**: not determined

The values are the means of the three independent assays.

Table 2: Susceptibility of enterotoxigenic *Escherichia coli* isolates to various antimicrobials in the absence or in the presence of an efflux pump inhibitor, PAβN.

Strains	MIC (µg/mL) chloramphenicol	Ratio of chloramphenicol accumulation at time 600s with and without CCCP*
20	512	72
30	512	33
39	1	2
40	256	45
48	128	5
50	> 512	50
73	128	45
75	256	37
80	1	3.5
98	128	2.8

CCCP*, energy uncoupler used at 50µM ; n.d**, not determined

The values are the means of the two independent assays.

Table 3: Antimicrobial susceptibility to chloramphenicol and chloramphenicol accumulation.

FIGURES

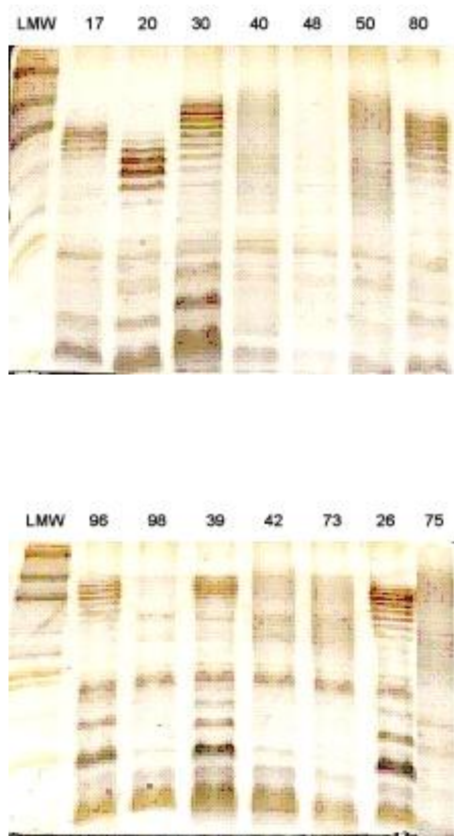

Fig. 1: Fig. 1: SDS-PAGE analysis of Lipopolysaccharide profiles in the fourteen *E. coli* strains. LPSs were prepared and separated by SDS-PAGE according to Thiolas *et al* [44]. LMW was the molecular weight standard.

Fig. 2: Uptake of chloramphenicol by *E. coli* isolates 39, 20, 50 and 75. The intracellular accumulation of [^{14}C]-radiolabeled chloramphenicol was carried out at various times in the absence (diamond) or in the presence (square) of CCCP according to the method previously described [19]. Values (expressed as counts per minute/optical density of cell suspension) were obtained from two independent experiments.

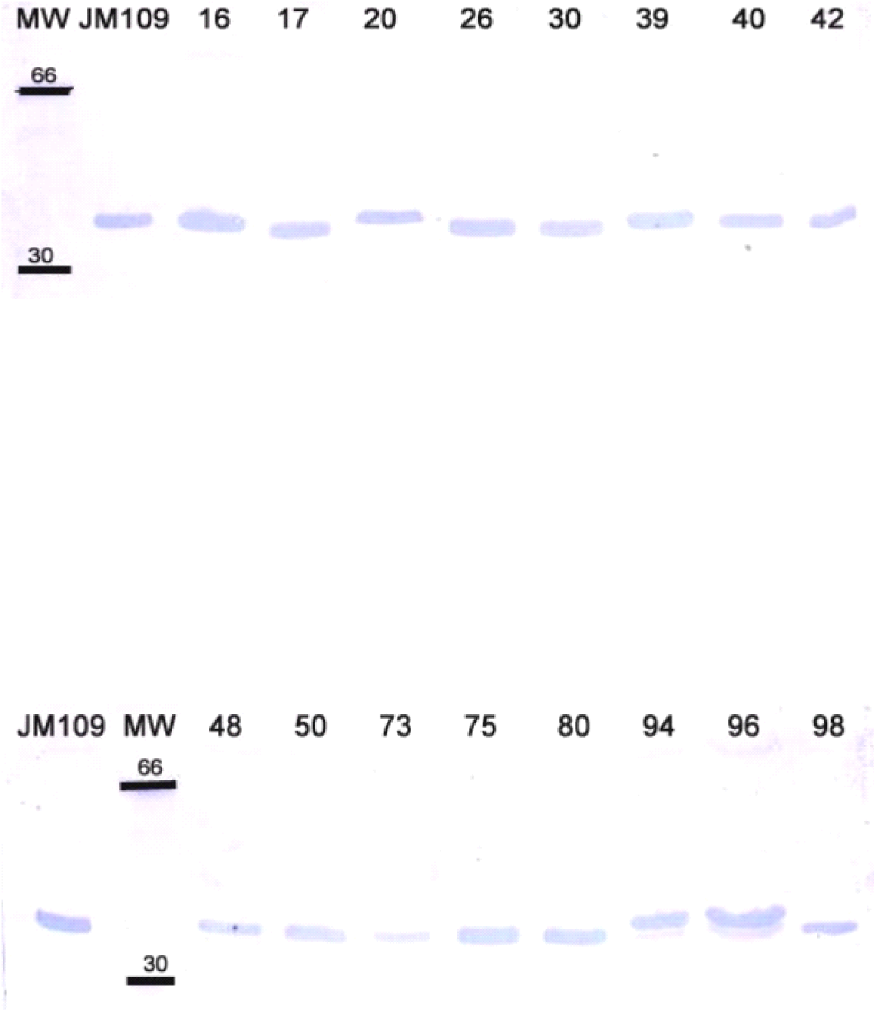

Fig. 3: Immunodetection of OmpF in *E. coli* strains . After SDS-PAGE of whole total proteins and electrotransfert on nitrocellulose sheets, the immunodetection assay were carried out with antibodies directed against OmpC and OmpF [30].

MW indicated the molecular weight standards (in kilodaltons). *E. coli* JM 109 was used as the control strain. Only the relevant part of the immunoblot is shown.