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Prevalence and characteristics of quinolone resistance in *Escherichia coli* in veal calves

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ABSTRACT

Quinolone resistance is studied and reported increasingly in isolates from humans, food-producing animals and companion animals. Resistance can be caused by chromosomal mutations in topoisomerase genes, plasmid-mediated resistance genes, and active transport through efflux pumps. Cross sectional data on quinolone resistance mechanisms in non-pathogenic bacteria from healthy veal calves is limited. The purpose of this study was to determine the prevalence and characteristics of quinolone resistance mechanisms in *Escherichia coli* isolates from veal calves, after more than 20 years of quinolone usage in veal calves. MIC values were determined for all isolates collected as part of a national surveillance program on antimicrobial resistance in commensal bacteria in food-producing animals in The Netherlands. From the strains collected from veal calves in 2007 ($n = 175$) all isolates with ciprofloxacin MIC $\geq$ 0.125 mg/L ($n = 25$) were selected for this study, and screened for the presence of known quinolone resistance determinants. In this selection only chromosomal mutations in the topoisomerase type II and IV genes were detected. The number of mutations found per isolate correlated with an increasing ciprofloxacin MIC. No plasmid-mediated quinolone resistance genes were found. The contribution of efflux pumps varied from no contribution to a 16-fold increase in susceptibility. No correlation was found with the presence of resistance genes of other antimicrobial classes, even though all quinolone non-wild type isolates were resistant to 3 or more classes of antibiotics other than quinolones. Over twenty years of quinolone usage in veal calves in The Netherlands did not result in a widespread occurrence of plasmid-mediated quinolone resistance, limiting the transmission of quinolone resistance to clonal distribution.

1. Introduction

Quinolones are considered to be drugs of critical importance to humans. They are also frequently used in veterinary medicine. Quinolones have been used in calves in The Netherlands for therapy of gastro-intestinal and respiratory infections since flumequine was approved in 1981. Fluoroquinolones have been introduced in 1987 (enrofloxacin) for therapeutic use in calves and poultry. In the 1990s other fluoroquinolones besides enrofloxacin have been approved for use in food-producing animals (e.g. marbofloxacin, danofloxacin). Although resistance against fluoroquinolones in microorganisms with a zoonotic potential poses a risk to human health, knowledge of the emergence of quinolone resistance mechanisms in non-pathogenic bacterial isolates from veal calves in specific is limited. Unlike poultry, dairy cattle and to a lesser extent slaughter pigs, veal calf herds in The

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Netherlands are assembled from animals imported from many different European countries. Therefore herds act as a large melting pot for animals with different antimicrobial resistances obtained from different (foreign) farms or during transport.

Antimicrobial resistance in food-producing animals in The Netherlands has been monitored since 1996 (Mevius et al., 2000). To study the effects of selection pressure of antibiotic usage in animals, the resistance level of commensal organisms was determined. Commensal *Escherichia coli* was isolated from the intestinal tract of the animals. These surveillance data show that the prevalence of multi-resistant *E. coli* (resistant to 3 or more classes of therapeutic antibiotics) in food-producing animals increased in the last decade (Mevius et al., 2010). In veal calves the prevalence of multi-resistant *E. coli* is stable since 2006 (Mevius et al., 2010). In veal calf *E. coli* isolates from 1996 to 2010, the prevalence of non-wild type phenotype to fluoroquinolones varies between 15 and 25%.

Quinolones target the topoisomerase type II and IV enzymes (Gellert et al., 1977; Kato et al., 1992). Quinolone resistance may arise through different possible mechanisms, of which various point mutations in the quinolone resistance determining region (QRDR) of the topoisomerase type II and IV enzymes are most frequently observed (Hooper, 2001). Plasmid-mediated quinolone resistance (PMQR) mechanisms however, are studied and reported increasingly. These mechanisms include *qnr* genes (Rodriguez-Martinez et al., 2011), *qepA* (Yamane et al., 2007), *oqxAB* (Hansen et al., 2007) and *aac(6′)-Ib-cr* (Robicsek et al., 2006) all result in reduced susceptibility to quinolones.

Finally, quinolone susceptibility can be decreased by altered permeation of the outer membrane and/or active efflux of drugs. For *E. coli* it was shown that the *marRAB* regulon is involved in both the regulation of outer membrane porin OmpF as well as the regulation of the endogenous AcrAB membrane efflux pump (Alekshun and Levy, 1997). Both play a role in regulation of the cytosolic concentration of certain antimicrobials, including quinolones.

The purpose of this paper was to determine the prevalence and molecular characteristics of quinolone resistance determinants in *E. coli* from veal calves, based on isolates from a national surveillance program, after more than 20 years of usage of quinolones in veterinary medicine in The Netherlands.

2. Materials and methods

2.1. Strain selection

All *E. coli* isolates were obtained from randomly collected pooled fecal samples (one *E. coli* per pooled sample). Each pooled sample was taken from one herd per farm as part of the annual surveillance program on antimicrobial resistance in animals in The Netherlands. The complete 2007 strain collection, stored at −80°C, includes 175 *E. coli* isolates obtained from veal calves. From all 175 isolates MICs were determined for a customized panel of antibiotics (see Section 2.2). From this collection, all non-wild type isolates with ciprofloxacin MIC ≥ 0.125 mg/L (designated 'quinolone non-wild type (WT) selection') were included in this study ($n = 25$) for further analysis.

2.2. Antimicrobial susceptibility

Susceptibility to antimicrobials was determined by broth microdilution according to ISO standards (ISO 20776-1:2006), using microtiter trays with a customized panel of dehydrated antibiotics. This panel included sulfamethoxazole, gentamicin, ampicillin, cefotaxime, ceftazidime, tetracycline, streptomycin, trimethoprim, ciprofloxacin, nalidixic acid, chloramphenicol, florfenicol, kanamycin and colistin (panel EUMVS, Sensititre©, Trek Diagnostics, UK).

2.3. Sequence analysis QRDR & PMQR

The twenty five selected quinolone-resistant isolates were screened for the presence of mutations in the QRDR region of *gyrA*, *gyrB*, *parC* and *parE* as described before (Everett et al., 1996), using *E. coli* ATCC 25922 as a quinolone susceptible control strain.

The presence of PMQR genes was determined as described previously for *qnrA* and *qnrS* (Cavaco et al., 2008a), *qnrB* (Cattoir et al., 2007), *qnrC* (Wang et al., 2009) and *qnrD* (Cavaco et al., 2009), *qepA* (Richter et al., 2010) and *aac(6′)-Ib-cr* (Park et al., 2006). All sequence reactions were performed using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Since the OqxAB efflux pump is not specific to quinolones, its presence was not determined. However, the contribution of efflux pumps in general was determined by a separate assay.

2.4. Contribution of efflux pumps

For each isolate of the quinolone non-WT selection with a ciprofloxacin MIC of 0.25–2 mg/L growth curves were determined at the following ciprofloxacin (Sigma–Aldrich, Germany) concentrations: 0 (negative control), 0.06, 0.125, 0.25, 0.5, 1 and 2 mg/L. Isolates with ciprofloxacin MIC > 8 mg/L were exposed to ciprofloxacin concentrations of 0, 0.5, 1, 2, 4, 8 and 16 mg/L. To determine the contribution of efflux pumps to quinolone susceptibility, all growth curves were measured with or without the presence of efflux pump inhibitors 1-(1-naphthylmethyl)-piperazine (NMP) (Sigma–Aldrich, Germany) or phenylalanine-arginine-ß-naphthylamide (PAßN) (Sigma–Aldrich, Germany), both at a concentration of 50 mg/L. A cell suspension of 1.5×10^6 CFU/mL in cation adjusted Mueller Hinton broth (CAMHB, Sensititre©, Trek Diagnostics, UK) was prepared for each isolate. Growth was determined by measuring optic density (OD) of 200 μL cell suspension with different ciprofloxacin concentrations with and without an efflux pump inhibitor. Samples were measured at 37 °C in a honeycomb multiwell plate, using a wide band filter (420–580 nm) with 10 min intervals for 7 h, using the Bioscreen C (Oy Growth Curves AB Ltd, Finland). All measurements were performed in triplicate. The area under the curve (AUC) was determined for all growth

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curves using equation 1 (OECD, 1984). One-way analysis of variance (ANOVA) was performed separately on the three treatments with PAßN, NMP or no EPI respectively, to determine at which ciprofloxacin concentration growth was significantly reduced using 0 mg/L ciprofloxacin as negative control. Furthermore, the negative control curves of PAßN, NMP and no inhibitor were also compared at the significance level $p = 0.05$.

Calculation of area under the curve:

$$\mathrm{AUC} = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_0 - 2N_0}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1}) \quad (1)$$

AUC is the area under curve; N_0 is the OD of cell suspension at time t_0; N_1 is the OD of cell suspension at time t_1; N_n is the OD of cell suspension at time t_n; t_1 is the time of first measurement after beginning of test; t_n is the time of nth measurement after beginning the test.

2.5. Micro array analysis

To determine the presence of antimicrobial resistance genes of other classes of antibiotics than quinolones, all twenty five isolates from the quinolone non-WT selection were screened using the Amr-ve kit on a miniaturized array platform (Alere, UK). Cell lysis, target labeling, and probe hybridization were performed according to the manufacturers' protocol.

3. Results

3.1. Antimicrobial resistance phenotype

For the total 2007 strain collection ($n = 175$), non-wild type MICs were determined for ampicillin, cefotaxime, ceftazidime, gentamicin, tetracycline, sulfamethoxazole, trimethoprim, florfenicol, chloramphenicol, streptomycin, kanamycin, ciprofloxacin and nalidixic acid (Table 1). Non-wild type MICs for colistin were not observed. From this collection, twenty five isolates showed a non-wild type MIC for ciprofloxacin, ranging from 0.25 to >8 mg/L, and for nalidixic acid >64 mg/L (Tables 1 and 2). In these twenty five isolates, designated "quinolone non-WT selection", the percentage of non-wild type MICs for the other antibiotics in the above mentioned panel was 1.5–4 fold higher compared to the total strain collection (Table 1). As a result, isolates with a non-WT quinolone phenotype were co-resistant to at least 3 additional classes of antimicrobials other than (fluoro)quinolones (penicillins, cephalosporins, tetracyclines, aminoglycosides, sulfonamides or phenicols) (Fig. 1).

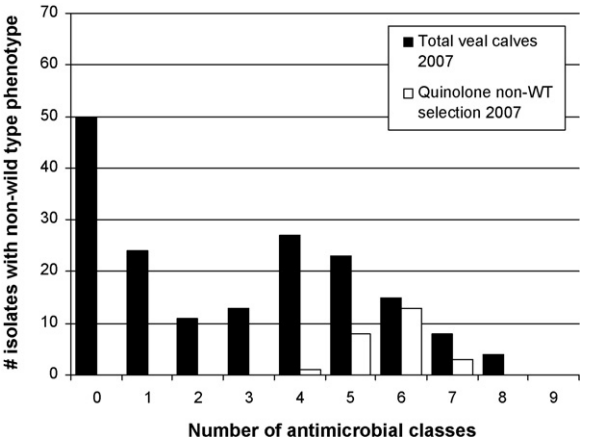

Fig. 1. Number of antimicrobial classes to which *E. coli* strains from the present study show non-wild type MIC-values.

3.2. Mutations in QRDR

All isolates harboured one or more point mutations in the QRDR. A clear correlation was shown between the increasing MICs of ciprofloxacin and the number of mutations found in the QRDR region of primarily *gyrA* and in addition *parC* and *parE* (Table 2). No mutations were found in *gyrB*. Isolates with a ciprofloxacin MIC up to 1 mg/L showed only one point mutation in *gyrA*, except for isolate 43.72, which has a MIC of 0.5 mg/L and also harboured a point mutation in *parE*. Isolate 41.25, with an additional mutation in *parC*, had a ciprofloxacin MIC of 2 mg/L. All remaining isolates, having ciprofloxacin MIC-values >8 mg/L, harboured two mutations in the the *gyrA* gene and an additional mutation in *parC*. In addition, one mutation in *parE* was observed in three of the highly resistant isolates.

3.3. Plasmid-mediated quinolone resistance

All isolates were screened for the presence of PMQR mechanisms. No genes were found belonging to the *qnr* gene family, nor the quinolone specific efflux pump *qepA*. One isolate harboured an *aac(6′)-1b gene*, however it did not show the point mutation specific for the modified aminoglycoside acetyltransferase variant *aac(6′)-1b-cr*.

Table 1
Percentage of non-wild type MICs in both '2007 total strain collection' and 'quinolone non-WT selection'.

	CIP	NAL	AMP	CTX	CTZ	GEN	TET	SUL	TMP	FFN	CHL	STR	KAN	COL
Total strain collection ($n = 175$)	14	14	47	5	3	7	70	51	42	10	28	52*	24*	0*
Quinolone non-WT selection ($n = 25$)	100	100	84	4	12	36	100	100	88	40	76	88	68	0

CIP, ciprofloxacin; NAL, nalidixic acid; AMP, ampicillin; CTX, cefotaxime; CTZ, ceftazidime; GEN, gentamicin; TET, tetracyclin; SUL, sulfamethoxazole; TMP, trimethoprim; FFN, florfenicol; CHL, chloramphenicol; STR, streptomycin; KAN, kanamycin; COL, colistin.
* $n = 75$ for these antibiotics.

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Table 2
Mutations in QRDR-region of topoisomerase II and IV genes and the presence of resistance genes to antibiotics other than quinolones.

Isolate	MIC mg/L		Mutations in QRDR				Resistance genes of other antimicrobial classes[e]
	CIP[a]	NAL[a]	*gyrA*[b]	*gyrB*	*parC*[c]	*parE*[d]	
37.22	0.25	>64	S83L	–	–	–	*aadA1*, *floR*, bla_{OXA-1}, *tet*(B)
42.50	0.25	>64	D87N	–	–	–	*aadA1*, *dfrA1*, *sul3*, bla_{TEM-1}
42.57	0.25	>64	S83L	–	–	–	*dfrA17*, *sul2*, bla_{TEM-1}, *tet*(B)
43.64	0.25	>64	S83L	–	–	–	*aadA1*, *catA1*, *dfrA1*, *sul2*, *tet*(B), bla_{TEM-1}, *strB*
44.19	0.25	>64	S83L	–	–	–	*aadA1*, *catA1*, *dfrA1*, *tet*(A), bla_{TEM-1}, *sul1*, *strB*
44.36	0.25	>64	S83L	–	–	–	*aadA1*, *tet*(A), *dfrA1*, *sul1*
37.11	0.5	>64	S83L	–	–	–	*aadA1*, *aadA2*, *cmlA1*, *sul3*, *catA1*, *dfrA1*
40.75	0.5	>64	S83L	–	–	–	*aadA1*, *dfrA1*, *sul3*, bla_{TEM-1}
41.67	0.5	>64	S83L	–	–	–	*sul2*, *tet*(A), bla_{TEM-1}, *strB*
42.09	0.5	>64	S83L	–	–	–	*aadA1*, *cmlA1*, *sul3*, bla_{TEM-1}
42.52	0.5	>64	S83L	–	–	–	*aadA1*, *dfrA1*, *strB*
43.72	0.5	>64	S83L	–	–	S458A	*aadA1*, *aadA2*, *catA1*, *cmlA1*, *dfrA1*, *sul1*, *sul2*, *sul3*, bla_{TEM-1}, *tet*(A), *strA*, *strB*
45.01	0.5	>64	S83L	–	–	–	*aadA1*, bla_{TEM-1}, *sul1*, *sul2*, *tet*(A), *floR*, *dfrA1*, *strB*
40.10	1	>64	S83L	–	–	–	*aadA1*, *catA1*, *dfrA1*, bla_{TEM-1}, *tetB*
41.25	2	>64	S83L	–	S80R	–	*aadA1*, *aadA2*, *catA1*, *dfrA1*, *sul3*, bla_{TEM-1}, *tetB*
37.38	>8	>64	S83L; D87N	–	S80I	–	*catA1*, *sul2*, bla_{TEM-1}, *tet*(A), *strA*, *strB*
40.07	>8	>64	S83L; D87N	–	S80I	S458A	*aadA1*, *aadA2*, *catA1*, *dfrA1*, *tet*(A), *tet*(B), *strB*
40.79	>8	>64	S83L; D87Y	–	S80I	S458A	*aac6Ib*, *catA1*, *dfrA1*, bla_{TEM-1}, *tet*(B)
41.26	>8	>64	S83L; D87N	–	E84K	–	*aadA1*, *catA1*, *dfrA1*, *sul1*, *tet*(A), bla_{TEM-1}, *strB*
41.59	>8	>64	S83L; D87N	–	S80I	–	*aadA1*, *dfrA1*, *floR*, *sul1*, *sul2*, bla_{TEM-1}, *tet*(B)
41.70	>8	>64	S83L; D87N	–	S80I	–	*dfrA7*, *dfrA17*, *sul1*, *sul2*, *tet*(A), *strB*
42.03	>8	>64	S83L; D87N	–	S80I	–	*aadA1*, *catA1*, *dfrA1*, *sul1*, *sul2*, *tet*(A), bla_{TEM-1}, *strA*, *strB*
42.31	>8	>64	S83L; D87N	–	S80I	–	*aadA1*, *dfrA1*, bla_{TEM-1}, *strB*
42.53	>8	>64	S83L; D87N	–	S80I	–	*aadA1*, *aadA2*, *catA1*, *cmlA1*, *dfrA1*, *sul1*, *sul3*, bla_{TEM-1}, *tet*(A), *strA*, *strB*
43.47	>8	>64	S83L; D87N	–	S80I	S458A	*sul2*, *tet*(B), *dfrA14*, bla_{TEM-1}, *strA*, *strB*

[a] CIP: ciprofloxacine, NAL: nalidixic acid.
[b] S83L, substitution of serine to leucine at amino acid 83; D87N, aspartic acid to asparagine; D87Y, aspartic acid to tyrosine.
[c] S80R, serine to arginine; S80I, serine to isoleucine; E84K, glutamic acid to lysine.
[d] S458A, serine to alanine.
[e] Resistance determinants detected using the Identibac AMR-ve array tubes. The probe for bla_{OXA-1} does not distinguish between $bla_{OXA-1, -30, -31}$ and $_{-33}$. The probe for *cmlA1*does not distinguish between *cmlA1*, *-4*, *-5*, *-6* and *-7*. The probe for bla_{TEM-1}detects all bla_{TEM} subtypes.

3.4. Contribution of efflux pumps

The contribution of efflux pumps to the susceptibility of isolates to fluoroquinolones was determined by measuring growth inhibition at ciprofloxacin concentrations varying from 0.06 to 16 mg/L, with or without the presence of efflux pump inhibitors. Growth inhibition was quantified by calculating the AUC at each ciprofloxacin concentration. All AUCs were compared to a negative control (0 mg/L ciprofloxacin) using the same inhibitor. As demonstrated in Table 3, in all isolates with a ciprofloxacin MIC up to 2 mg/L, without addition of an inhibitor, significant growth reduction occurred at 2 or 3 dilution steps below the MIC value. For all isolates with MIC > 8 mg/L, significant growth reduction without inhibitor was observed at various ciprofloxacin concentrations ranging from 1 to 8 mg/L. When adding inhibitor NMP to the medium, no shift in susceptibility could be observed for 11 out of 13 isolates with a MIC up to 0.5 mg/L, since these strains were also growth inhibited at 0.06 mg/L without addition of an inhibitor. Furthermore, adding NMP to isolates with MIC > 0.5 mg/L resulted in no effect for three isolates and an increase in ciprofloxacin susceptibility of 1–4 dilution steps in all remaining isolates. When adding inhibitor PAßN to the medium, isolates with a MIC up to 0.5 mg/L showed similar results compared to the addition of NMP. Adding PAßN to isolates with MIC > 0.5 mg/L resulted in no effect for five isolates and an increase in ciprofloxacin susceptibility of 1–3 dilution steps for the remaining isolates. Comparing the negative controls (0 mg/L ciprofloxacin) of the treatments without an inhibitor, to the treatments with either NMP or PAßN, shows that adding an inhibitor without ciprofloxacin also causes some growth reduction. In medium supplemented with NMP, 24 out of 25 isolates show a reduction in growth of less than 25% compared to the medium lacking an inhibitor. With PAßN 15 out of 25 isolates show a growth reduction of less than 25%, the remaining 10 exceed 25%.

3.5. Micro array analysis

As shown in Table 2, all quinolone non-WT isolates harboured genes for resistance to three or more classes of antibiotics other than quinolones. The number of resistance genes detected for each isolate varied from 3 to 12. Most abundant determinants detected were the aminoglycoside resistance gene *aadA1* (75% of the isolates), single or multiple variants per isolate of trimethoprim resistance gene *dfr* (83%), one or more variants per isolate of the sulfamethoxazole resistance gene *sul* (75%) and ß-lactamase bla_{TEM} (75%). No *qnr* genes were detected using the array. The gene *qepA* was not present on the chip. One *aac(6′)-1b* gene was detected, however as mentioned above, it did not show the point mutation specific for the

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Table 3
Ciprofloxacin concentrations at which growth was significantly reduced.

Isolate	MIC (mg/L)	Growth inhibition at [ciprofloxacin]* (mg/L)		
		No inhibitor	NMP	PAßN
37.22	0.25	0.06	0.06	0.06
42.50	0.25	0.06	0.06	0.06
42.57	0.25	0.06	0.06	0.06
43.64	0.25	0.06	0.06	0.06
44.19	0.25	0.06	0.06	0.06
44.36	0.25	0.06	0.06	0.06
37.11	0.5	0.06	0.06	0.06
40.75	0.5	0.06	0.06	0.06
41.67	0.5	0.06	0.06	0.06
42.09	0.5	0.06	0.06	0.06
42.52	0.5	0.12	0.12	0.06
43.72	0.5	0.06	0.06	0.06
45.01	0.5	0.12	0.06	0.06
40.10	1	0.12	0.06	0.06
41.25	2	0.06	0.12	0.06
37.38	>8	4	2	1
40.07	>8	8	4	2
40.79	>8	4	2	8
41.26	>8	8	0.5	2
41.59	>8	1	0.5	2
41.70	>8	2	2	0.5
42.03	>8	4	2	2
42.31	>8	4	1	8
42.53	>8	4	1	8
43.47	>8	8	8	2

* Lowest concentration of ciprofloxacin at which significant growth inhibition was observed ($p \leq 0.05$) compared to the control (same isolate, same inhibitor, 0 mg/L ciprofloxacin added). Growth curves of all isolates were determined in triplicate and quantified by calculating the 'area under the curve' of each growth curve using Eq. (1). One-way ANOVA was used to determine significant reduction in growth.

aac(6′)-1b-cr variant. Furthermore, in 14 isolates *str* genes were detected, of which 5 were positive for both *strA* and *strB*, and 9 were only positive for *strB*. Since the presence of only *strB* is rather uncommon, this was verified by PCR. The PCR results show that all isolates harbouring *strB* also harbour *strA* (data not shown), indicating the array showed several false negative results for *strA*.

4. Discussion

Resistance levels of *E. coli* to (fluoro)quinolones in veal calves in The Netherlands have leveled off to values between 15 and 25 percent (Mevius et al., 2010). Compared to several other European countries, this quinolone resistance level is relatively high (EFSA, 2010). The Dutch resistance level of ciprofloxacin and nalidixic acid in *E. coli* from bovine sources is similar to France and Italy, although the EFSA report is based on data of cattle and not specifically veal calves. One of the causes of this relatively high resistance level might be the usage of antimicrobials. In The Netherlands and France both the overall usage of antibiotics and the usage of fluoroquinolones in veterinary medicine is two to ten times higher compared to other European countries (Grave et al., 2010).

The first quinolone resistance mechanism discovered was mutation of the gyrase genes (Gellert et al., 1977). In 1998 the first *qnr* gene was discovered (Martinez-Martinez et al., 1998), followed in the last decade by more *qnr* variants, *qepA* and *aac(6′)Ib-cr*, all plasmid-mediated genes specific for quinolone resistance. Even though PMQR mechanisms are studied and reported increasingly, the results in this study show that after screening all quinolone non-WT *E. coli* isolates from 2007, the reduced susceptibility to quinolones was due to chromosomal mutations in the QRDR region. The mutations found in *gyrA*, *parC* and *parE* in this selection (Table 2) have been reported previously (Everett et al., 1996; Sorlozano et al., 2007). No mutations were observed in the *gyrB* gene.

No plasmid-mediated quinolone resistance determinants were found in this selection of strains. Recently, in an ongoing epidemiological study determining prevalence of non-WT antibiotic phenotype on 50 farms (Bosman; unpublished data) one *qnrB19* gene was detected in veal calves in The Netherlands (Hordijk et al., 2011) and one *qnrS1* gene was observed in veal calves in the annual surveillance program on antimicrobial resistance in animals in The Netherlands (Veldman, unpublished data, 2010), indicating that *qnr* prevalence is still low. This is consistent with observations in other food-producing animals in the Netherlands. So far, several *qnr* genes were detected in *Salmonella enterica* and *E. coli* (Veldman et al., 2008, 2011), all isolated from poultry. No *qepA* or *aac(6′)-Ib-cr* have been detected, indicating that the prevalence is also very low. This low prevalence indicates that the transmission of quinolone resistance within and between animals is limited to clonal spread. In surrounding European countries plasmid-mediated quinolone resistance in food-producing animals has been observed more frequently, however available data is still limited (Cavaco et al., 2008b; Kehrenberg et al., 2006; Kirchner et al., 2011; Veldman et al., 2011). Outside Europe there is a higher prevalence of PMQR determinants in food-producing animals (Huang et al., 2009; Ma et al., 2008; Yue et al., 2008). However, in all studies reported outside The Netherlands, only Kirchner et al. report a *qnrS1* isolated from cattle. Veal calves at Dutch fattening farms originate from different dairy farms, including dairy farms from other EU-countries. Therefore, a continuous mixture of bacterial flora will occur in veal calf production. Due to selection pressure by usage of antibiotics, ideal circumstances for selection of resistant organisms are present. Still in veal calves to date, PMQR has been detected only two times, indicating that in cattle in the EU PMQR is still virtually absent.

Data presented in Table 3 shows that all isolates with a ciprofloxacin MIC > 1 mg/L and two isolates with a MIC of 0.5 mg/L become more susceptible to ciprofloxacin in the presence of an efflux pump inhibitor. These isolates with a detectable reduction in susceptibility (14 isolates) respond differently to the addition of an inhibitor. Four isolates only showed a reduction when adding NMP, three with PAßN and seven in response to both. The exact mechanism of efflux pump inhibitors is still unknown, although NMP has shown to reverse multi-drug resistance in *E. coli* overexpressing resistance-nodulation-cell division (RND) type efflux pumps, but not in pump deficient mutants. PAßN also shows inhibitory effects without the presence of RND type efflux pumps in *E. coli* (Bohnert and Kern, 2005). *E. coli*

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is known to harbour different efflux pumps, of which the RND efflux pump AcrAB associated with TolC is most predominant and best characterized (Kumar and Schweizer, 2005). The difference in response to the addition of an inhibitor suggests that these isolates differ in expression of different efflux pumps, possess different mutations in their efflux pumps or regulator genes, have differences in efflux pump transcription level, or differ in number of copies of membrane porins (Kumar and Schweizer, 2005).

Efflux pump inhibitors NMP and PAßN are commonly used inhibitors in various concentrations ranging from 20 to 160 mg/L (Kern et al., 2006; Saenz et al., 2004). This study shows that growth is significantly reduced in all isolates, solely by adding an inhibitor to the medium without ciprofloxacin. One isolate showed only a reduction in growth by adding NMP without ciprofloxacin and not by adding PAßN. Therefore, the observed mild effect of increased susceptibility by adding inhibitors is indicative, not conclusive. Some studies have shown that the MIC values for the inhibitors themselves is lower in wild type strains than efflux pump deficient strains (Kern et al., 2006; Saenz et al., 2004), suggesting that the inhibitors themselves may also act as substrate for efflux. Speculating on the results in the present study, the mild increase in susceptibility by adding an inhibitor to the medium and the growth inhibition in all isolates by just adding 50 mg/L NMP or PAßN without ciprofloxacin, suggests that there is no overexpression of efflux pumps. However, this is not confirmed by quantitative PCR.

All twenty five selected isolates in this study were resistant to 3 or more classes of antibiotics other than quinolones. Even though some isolates show similarities, there is great diversity in the presence of resistance determinants between isolates (Table 2). Since quinolone resistance in these isolates is mainly caused by mutations in the chromosomal topoisomerase genes, there is no direct linkage between quinolone resistance and the presence of other resistance determinants. However, looking at the 'quinolone non-WT selection' in Table 1, the percentages of resistant isolates for all antibiotics tested except colistin are higher than the percentages in the 'total strain collection'. This suggests that co-selection of resistance determinants for antibiotics other than quinolones occurred.

In conclusion, *E. coli* from Dutch veal calves are frequently resistant to quinolones. In the sample selection described in this study quinolone resistance is mainly driven by chromosomal mutations in the QRDR region of *gyrA/B* and *parC/E*. No PMQR determinants were detected.

Inhibition of efflux pumps with NMP or PAßN resulted in a mild decrease in MIC values, indicating a limited contribution of efflux pumps to reduced susceptibility to quinolones in the tested isolates.

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