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REGULAR ARTICLES

High level of multidrug-resistant *Escherichia coli* in young dairy calves in southern Vietnam

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Abstract

This study investigated the occurrence of antimicrobial-resistant *Escherichia coli* in dairy calves in southern Vietnam. Fecal samples were taken directly from the rectum of 84 calves from 41 smallholder dairy farms, when newborn and at 14 days of age for isolation of *E. coli. Escherichia coli* strains were isolated from 144 of the 168 fecal samples tested. Of the 144 *E. coli* isolates, 40% were found to be susceptible to all 12 antimicrobial drugs tested and 53% of the *E. coli* isolates were resistant to at least three antimicrobials. Calves were colonized with antimicrobial-resistant *E. coli* already on the day of birth. Resistance to tetracycline was most common, followed by resistance to sulfamethoxazole, ampicillin, trimethoprim, and ciprofloxacin. Four isolates carried a gene encoding for extended-spectrum cephalosporinases (ESC), and these genes belonged to $bla_{\text{CTX-M}}$ group 1 (2 isolates), $bla_{\text{CTX-M}}$ group 9 (1 isolate), and $bla_{CMY\text{-}2}$ (1 isolate). Thirty-three isolates had a plasmid-mediated quinolone resistance (PMQR) phenotype, and 30 of these carried the *qnr*S gene. These results are of importance for management routines of dairy cattle to prevent the spread of antimicrobial resistance.

Keywords Antimicrobial resistance · Extended-spectrum cephalosporinases · Plasmid-mediated quinolone resistance

Introduction

The emergence and spread of antimicrobial-resistant bacteria is an increasing problem and a threat to global public health (WHO 2017). Due to the development of antimicrobial resistance, the European Union banned the use of growth-promoting antimicrobials in animal production in 2006 (European Council 2006). The Ministry of Agriculture and Rural Development (MARD) in Vietnam has had regulations banning some antimicrobials for growth-promoting purposes in animal production since 2002 (MARD 2002).

In Vietnam, 70% of the drug products used in animal production are antimicrobials (An 2009). Antimicrobial consumption has contributed to high levels of antimicrobial-resistant fecal bacteria in calves (Pereira et al. 2014). *Escherichia coli*, a commensal bacterium of the gastrointestinal tract in both animals and humans, are frequently used as an indicator to monitor antimicrobial resistance in fecal samples. The few publications available on the use of antimicrobials and problems with antimicrobial resistance in Southeast Asia (SE Asia) have primarily focused on pig, chicken, fish, and shrimp production (Nhung et al. 2016).

Resistant organisms or their genes can be transmitted from animals to humans by direct contact, via the food chain, or through environmental contamination (Iglesias et al. 2012) and are therefore of great concern for human health. Production of extended-spectrum cephalosporinases (ESC) is one of the most common mechanisms of oxyiminocephalosporin resistance in *E. coli* (Pitout and Laupland 2008). The $bla_{CTX\text{-}M}$ gene has been detected in a large proportion of ESC-producing *E. coli* isolated from dairy calves, chicken meat, and pork (Le et al. 2015; Awosile et al. 2018). The prevalence of antimicrobial-resistant bacteria, especially quinolone-resistant bacteria in chickens, is higher in SE Asia countries (Indonesia, Thailand, and Vietnam) than in developed countries (Usui et al. 2014). Quinolones are critically important antimicrobials for treating severe infections in

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humans (WHO 2017), and reduced susceptibility to quinolone can lead to treatment failure and is considered a public health risk (Gagliotti et al. 2008). Holloway et al. (2017) reported that antimicrobial drugs are sold without prescription in SE Asia; these countries have high antimicrobial use and poor implementation of polices to promote suitable use. Based on the findings, it can be assumed that quinolone resistance and plasmid-mediated quinolone resistance (PMQR) are more common in dairy calves in SE Asia than in countries with more restrictive antimicrobial use. However, little information is available to date about the prevalence of antimicrobial resistance microbes in dairy calves in SE Asia.

The main aim of this study was therefore to investigate the occurrence of antimicrobial-resistant *E. coli* in samples from young dairy calves on smallholder dairy farms in southern Vietnam, with special emphasis on PMQR and ESC resistance because of their potential transfer to human pathogens.

Materials and methods

Farms and sampling

The study was conducted on 41 smallholder dairy farms located in Dong Nai province, southern Vietnam. A total of 84 dairy calves were available for sampling in the study period (August to December), from 5 to 10 dairy cows per farm and with at least two calves born during the period. Each farm was visited twice, with 14 days between the visits. Fecal samples were taken directly from the rectum of calves when newborn and at 14 days of age, and placed in sterile tubes. The tubes were immediately placed on ice and transferred to the laboratory for isolation of *E. coli*. In total, 168 fecal samples were collected.

Sample analysis

Escherichia coli culture and isolation

From each sample, fecal material was streaked onto MacConkey agar (Neogen, Michigan, USA) using a sterile cotton-tipped swab and incubated overnight at 37 °C. Five lactose-fermenting (bright pink) colonies with typical *E. coli* morphology were randomly selected from the MacConkey agar plate. These colonies were subcultured on horse blood agar plates (Neogen, Michigan, USA), incubated overnight at 37 °C, and tested for production of tryptophanase (indole) using the spot indole test (Miller and Wright 1982). Lactose- and indole-positive isolates with typical colony morphology (bright pink on MacConkey agar, blue in spot indole test and single-colony types) were considered *E. coli*. Confirmed isolates of *E. coli* were transferred to 2-mL microtubes (SARSTEDT, Nümbrecht, Germany) containing 0.5 mL serum broth supplemented with 15% glycerol. The microtubes were placed in a freezer at − 80 °C. One of the five frozen isolates of *E. coli* from each calf sample was selected at random and sent frozen (on dry ice) to the National Veterinary Institute, Uppsala, Sweden. Directly upon arrival, isolates were transferred to a freezer at − 20 °C and stored until further testing.

Antimicrobial susceptibility testing

For each isolate, the minimum inhibitory concentration (MIC) to 12 common antimicrobials was determined using broth microdilution. Testing was performed according to recommendations by the Clinical and Laboratory Institute (CLSI 2013) using VetMIC panels (National Veterinary Institute, Uppsala, Sweden) and cation-adjusted Mueller Hinton Broth (Becton Dickinson, Cockeysville, MD, USA). Epidemiological cut-off values (ECOFFs) set by the European Committee on Antimicrobial Susceptibility Testing were used to classify isolates as susceptible or resistant. Antimicrobials, ranges, and ECOFFs are given in Table 1. Quality control, using the reference strain *E. coli* ATCC 25922, was conducted in parallel with each batch of isolates and all results were within acceptable ranges.

Isolates with cefotaxime MIC > 0.25 μg/mL were phenotypically tested for production of ESC with broth microdilution using EUVSEC2 panels (Trek Diagnostic System, Oakwood Village, OH, USA) and cation-adjusted Mueller Hinton broth (Becton Dickinson, Cockeysville, MD, USA). Isolates with a cefotaxime MIC > 0.25 μg/mL or a ceftazidime MIC > 0.5 μg/mL on the EUVSEC2 panels were further screened by multiplex-polymerase chain reaction (PCR) for detection of the following gene groups: plasmid-mediated AmpC (pAmpC) and $bla_{\text{CTX-M}}$ (Pérez-Pérez and Hanson 2002). Isolates with ertapenem MIC > 0.06 μg/mL or meropenem MIC > 0.125 μg/mL were further characterized by whole genome sequencing (WGS) to identify transferable genes encoding for carbapenemases.

The DNA used for WGS was extracted from colonies on horse blood agar plates, using an EZ1 DNA tissue kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). The DNA concentration was determined using Qubit HS DNA-kit (Life Technologies, Carlsbad, CA, USA). Samples were submitted to GATC Biotech (Konstanz, Germany) and subjected to standard genomic library preparation followed by 2 × 150 bp paired-end sequencing using Illumina-based technology. To identify potential contamination and for confirmation of bacterial species, reads were checked using KRAKEN against the pre-built MiniKRAKEN 8GB database (2017-10-19) (Wood and Salzberg 2014). Reads were trimmed with Trimmomatic 0.36 and genome assembly was performed with SPAdes v.3.11.1 with the "- -careful" parameter and an input average

Table 1 Distribution of the minimum inhibitory concentration (MIC) of 12 common antimicrobials in fecal *E. coli* from newborn calves and calves aged 14 days (84 calves, 144 isolates)[1]

Antimicrobial	Resistance	Cut-off value	Distribution (%) of MIC (μg/mL)																	
			≤0.008	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>1024
Ampicillin	48.6%	8								16.0	32.6	2.8		1.4	2.1		3.4	41.7			
Cefotaxime	4.8%	0.25			12.5	78.5	4.2				1.3	3.5									
Chloramphenicol	34.0%	16									9.0	46.9	8.7	1.4	4.9	4.8	24.3				
Ciprofloxacin	40.3%	0.06		36.1	23.6		6.2	18.1	2.1	0.7	13.2										
Florfenicol	25.0%	16										38.9	33.3	2.8	1.4	23.6					
Gentamicin	9.8%	2						20.1	63.1	6.3	0.7	1.4	0.7	3.5	4.2						
Kanamycin	9.7%	8											90.3		9.7						
Nalidixic acid	20.8%	16								2.1	32.6	28.5	11.8	4.2	2.1	0.7	3.4	14.6			
Streptomycin	34.7%	16									11.8	36.2	10.4	6.9	6.2	4.9	9.0	9.0	5.6		
Sulfamethoxazole	49.3%	64											7.6	28.5	14.6					9.7	39.6
Tetracycline	57.6%	8								39.2	3.2			4.2	23.5	23.6	6.3				
Trimethoprim	43.8%	2					5.6	38.1	12.5			0.7			43.1						

[1] The shaded area represents range of antimicrobial tested

coverage of 50×, followed by Pilon v1.22 with default settings to correct assemblies (Bolger et al. 2014). Presence of potential genes encoding antibiotic resistance was checked using Antimicrobial Resistance Identification By Assembly (ARIBA) v2.11.1 (Hunt et al. 2017), together with the downloaded databases of CARD v2.0.1 and ResFinder (2018-06-07) (McArthur and Wright 2015).

Isolates with ciprofloxacin MIC > 0.06 μg/mL and nalidixic acid MIC < 32 μg/mL were selected for PCR detection of plasmid-mediated quinolone resistance (PMQR) genes. The screening for PMQR genes included *qnrA*, *qnrB*, *qnrS*, and *aac(6')-1b-cr*, using PCR assays described earlier (Cavaco et al. 2008).

Statistical analysis

Fisher's exact test was used to compare the observed proportions of resistance to each antimicrobial compound in isolates from samples obtained from calves aged 0 and 14 days. The significance level was set to $p < 0.05$. All statistical analyses were conducted in Stata 13 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX, USA).

Results

From the total of 168 fecal samples, *E. coli* was isolated in 144 samples and was not detected in 24 samples (21 samples from newborn calves, three from 14-day-old calves). These *E. coli* isolates were subjected to antimicrobial susceptibility testing, the results of which are presented in Table 1. Of the 144 *E. coli* isolates, 40% were found to be pan-susceptible (defined as susceptible to all antimicrobial drugs tested) and 53% were multidrug-resistant (defined as resistant to at least three antimicrobial drugs). Resistance to tetracycline was most common (57.6% of the isolates), followed by sulfamethoxazole (49.3%), ampicillin (48.6%), trimethoprim (43.8%), and ciprofloxacin (40.3%).

Seven isolates had a cefotaxime MIC > 0.25 μg/mL and were eligible for phenotypic ESC screening, and six of these were sent for genotypic screening based on the phenotypic results (Table 2). The phenotypic and genotypic screening confirmed that four isolates could be considered ESC producers. Two of these carried a gene belonging to $bla_{\text{CTX-M}}$ group 1 (from the same calf on two sampling occasions), another isolate carried a gene belonging to $bla_{\text{CTX-M}}$ group 9, and the fourth, a gene belonging to the pAmpC-producing gene group bla_{CMY} group 2. The two remaining isolates of the six screened also exceeded the ECOFFs for ertapenem and meropenem, and thus showed carbapenem resistance. However, no transferrable genes that could explain the carbapenem resistance phenotype were identified. The seventh isolate was below the cefotaxime and ceftazidime ECOFFs, and was thus not of interest for further screening.

Thirty-three isolates were eligible for PMQR screening, based on their resistance profile of ciprofloxacin and nalidixic acid, of which 26 were from samples collected at 14 days of age. Thirty of the screened isolates (21% of all isolates) carried the *qnrS* gene, but none of the other genes, i.e., *qnrA*, *qnrB*, and *aac(6')-1b-cr*, was detected. The 30 PMQR-positive strains were from 28 calves located on 22 different farms.

Table 2 Results of phenotypic and genotypic screening for isolates producing extended-spectrum cephalosporinases (ESC) for seven isolates with cefotaxime MIC > 0.25 μg/mL obtained from feces from dairy calves. ECOFF, epidemiological cut-off value

	Antimicrobial									ESC gene group
	Cefepime	Cefotaxime	Cefotaxime and clavulanic acid	Cefoxitin	Ceftazidime	Ceftazidime and clavulanic acid	Ertapenem	Imipenem	Meropenem	Temocillin	
Range tested	0.06–32	0.25–64	0.06/4–64/4	0.5–64	0.25–128	0.12/4–128/4	0.015–2	0.12–16	0.03–16	4–128	
ECOFF	0.125	0.25	NA	8	0.5	NA	0.064	0.5	0.125	> 32	
ID no.											
E 11.1.1	4	64	≤ 0.06	2	4	≤ 0.12	≤ 0.015	≤ 0.12	≤ 0.03	4	*bla*CTX-M-1
E 11.1.2	4	32	≤ 0.06	4	4	≤ 0.12	≤ 0.015	≤ 0.12	≤ 0.03	4	*bla*CTX-M-1
E 14.2.2	≤ 0.06	4	1	8	4	2	≤ 0.015	≤ 0.12	≤ 0.03	2	*CIT*
E 21.2.1	32	> 64	> 64	> 64	> 128	> 128	> 2	1	4	> 128	No genes found
E 33.2.1	1	8	8	> 64	2	1	2	1	1	> 128	No genes found
E 42.2.1	≤ 0.06	≤ 0.25	≤ 0.06	2	≤ 0.25	≤ 0.12	≤ 0.015	≤ 0.12	≤ 0.03	4	ND
E 57.2.2	4	64	≤ 0.06	2	0.5	≤ 0.12	≤ 0.015	≤ 0.12	≤ 0.03	4	*bla*CTX-M-9

NA, not applicable; *ND*, not done

The proportions of antimicrobial-resistant *E. coli* in samples from the same calves when newborn and at 14 days of age are shown in Fig. 1. Significantly ($P < 0.05$), larger proportions of isolates from samples taken at 14 days of age were resistant to ampicillin, chloramphenicol, ciprofloxacin, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim. No significant difference was observed for the remaining antimicrobials tested.

Discussion

The incidence of antimicrobial resistance (around 60%) among the *E. coli* isolates in the present study was higher than reported previously in Sweden (Duse et al. 2015a). This is most likely the result of high use of antimicrobials such as beta-lactams, aminoglycosides, fluoroquinolones, and macrolides in Vietnam (Carrique-Mas et al. 2015).

The incidence of resistance to ampicillin, tetracycline, and sulfamethoxazole was 48.6–57.6% among the 144 isolates tested. These antimicrobial resistance types have been found in over 50% of *E. coli* isolates from pigs and chickens in SE Asia (Nhung et al. 2016). The high proportion of tetracycline and ampicillin resistance probably reflects the long history of using these antimicrobials for treatment and prophylactic purposes (Tadesse et al. 2012). Chloramphenicol is banned for use in animal feed (MARD 2002) in Vietnam. However, chloramphenicol resistance was still high (34.0% of the isolates) in the present study, which might be due to chloramphenicol still being used in animal production (Nhung et al. 2016). Moreover, thiamphenicol and florfenicol, which belong to the same family as chloramphenicol, are still permitted for use in livestock and aquaculture (MARD 2010) and chloramphenicol resistance is commonly co-selected with these antimicrobials (Harada et al. 2006).

The high proportion of multidrug-resistant strains found in the present study might be a result of the common use of combinations of antimicrobials and of broad-spectrum antimicrobials in this region, as discussed in a previous study (Ström et al. 2017). It could also be a case of co-selection (Harada et al. 2006). It was previously documented that resistant strains selected during an antimicrobial treatment last for a long time in the intestinal tract when this treatment ceases. Additionally, these resistant strains could modify animal health and can be transmitted to other animals, especially to their offspring and accompanying animals (Roca-Saavedra et al. 2018).

Antimicrobial-resistant strains of *E. coli* were observed in newborn calves in the present study. Antimicrobials and resistant organisms are spread to the environment through wastes (feces, slurry, and wastewater) from agricultural animals (da Costa et al. 2013). A high level of general antimicrobial use might lead to a high proportion of resistant strains in the

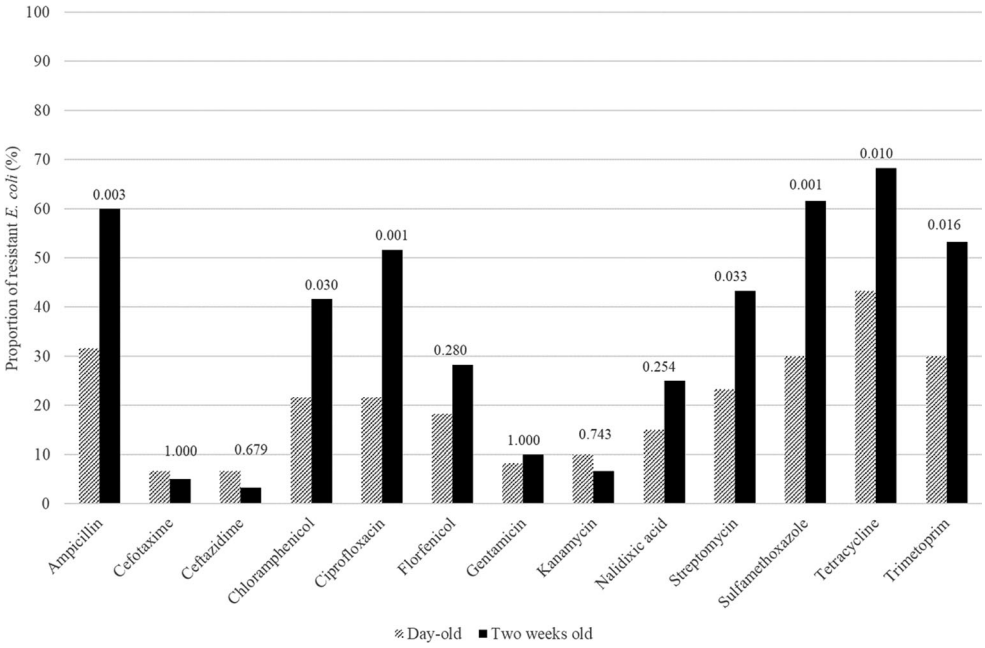

Fig. 1 Proportion of antimicrobial-resistant *Escherichia coli* sampled from feces from the same group of 60 calves on two occasions, when newborn and at 14 days old. *P* values indicate the results of Fisher's exact test on differences in the proportion of antimicrobial-resistant isolates at the two sampling occasions ($P < 0.05$ indicates significantly different proportions)

environment, and thus the *E. coli* that first enters the gastrointestinal tract of the calf is highly likely to be antimicrobial-resistant.

The proportions of ampicillin, chloramphenicol, ciprofloxacin, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim resistance were significantly lower in newborn calves than in the same calves at 14 days of age. Earlier studies have found that resistant strains dramatically colonize dairy calves after parturition, but that the prevalence of antimicrobial-resistant strains commonly peaks in calves at 14 days of age (Donaldson et al. 2006) and thereafter declines in calves aged 4–6 weeks (Berge et al. 2005). The reason is a transition from susceptible to resistant strains, and then back to susceptible strains, in the gut of the developing calf, and there is no reemergence of susceptible strains that have been dominant when the calves are younger (Hinton et al. 1985).

In the present study, ESC-producing *E. coli* was found in about 3% of the 144 isolates. However, only non-selective methods were used to detect ESC-producing isolates, so the true prevalence of ESC-producing *E. coli* is probably much higher. The ESC-producing gene groups ($bla_{\text{CTX-M}}$ and bla_{CMY}) found in the present study have been observed previously in Holstein dairy calves in Canada (Awosile et al. 2018). Moreover, $bla_{\text{CTX-M-1}}$, and $bla_{\text{CTX-M-9}}$ are the main β-lactamase resistance groups detected in *E. coli* isolates from chicken meat and pork in Vietnam (Le et al. 2015). Although two isolates showed phenotypic resistance to carbapenem, it was not possible to confirm this by genotypic characterization of these isolates after WGS. The reason could be that the gene encoding for carbapenem resistance was lost during repeated freezing and thawing cycles of the stored isolate, or that phenotypic resistance is encoded by genes not yet described. It could also be the case of a combination of chromosomal AmpC and porin loss.

The incidence of quinolone-resistant *E. coli* (QREC) isolates was 21%, although fluoroquinolone drugs are not used on the farms tested. One explanation could be the horizontal spread of PMQR genes from the surroundings, as the dairy farmers apply irrigation water, livestock wastewater, and manure to grass crops, possibly increasing the abundance of fluoroquinolones and antimicrobial resistance genes in the environment, as documented in recent studies (McKinney et al. 2018). There might also be some environmental cross-contamination of fluoroquinolones from human sanitary systems (Phu et al. 2016). The incidence of QREC was higher than reported previously in pre-weaned dairy calves (Duse et al. 2015b). A large proportion (91%) of the isolates eligible for PMQR screening showed the presence of PMQR genes, and to our knowledge, this is a unique finding in dairy calves in Vietnam. The *qnrS* gene has also been found in *E. coli* isolates from dairy calves in Canada (Awosile et al. 2018). Humans live in close proximity to calves in Vietnam, and thus, there is a high risk of these genes spreading to human pathogens, such as *Salmonella* (Veldman et al. 2011).

To our knowledge, this is the first report of antimicrobial resistance in dairy cattle and occurrence of ESC and *qnrS* genes in *E. coli* isolated from dairy calves in Vietnam. These findings should be taken into consideration by veterinarians/policy makers and by dairy farmers when devising management regimes for dairy cattle in order to combat antimicrobial resistance. They can also be used as a starting point for future antimicrobial resistance monitoring in Vietnam.

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Compliance with ethical standards

This study was performed with the consent of all farmers in the study. All handling of animals in connection with sampling was performed, considering animal welfare and following international and national guidelines.

Conflict of interest The authors declare that they have no competing interests.

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