Antimicrobial susceptibility of *Campylobacter cuniculorum* isolated from rabbits reared in intensive and rural farms

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Abstract

The present study aimed to investigate the antimicrobial susceptibility in *Campylobacter cuniculorum*. To do so, 29 isolates from rabbits reared in 18 intensive and 11 rural farms not epidemiologically correlated were tested. Minimum inhibitory concentration of 8 antimicrobial agents was determined using the agar dilution method recommended by the Clinical and Laboratory Standards Institute (Wayne, PA, USA), modified – for what supplements in the base medium and incubation conditions concern – for *C. cuniculorum* isolates. The isolates obtained from rural farming resulted susceptible to all the antimicrobial agents tested, with the exception of one isolate resistant to nalidixic acid. All the isolates obtained from intensively farmed rabbits were sensitive to chloramphenicol and ampicillin; 16 isolates were resistant to tetracycline; 15 to nalidixic acid and erythromycin; 13 and 10 isolates to ciprofloxacin and enrofloxacin, respectively; and only 1 to gentamicin. The resistance of several isolates to macrolides and fluoroquinolones, which are the drugs of choice in treatment of human campylobacteriosis, could pose a risk to human health if a pathogenic role of *C. cuniculorum* was demonstrated.

Introduction

Campylobacter spp., especially *C. jejuni* and *C. coli*, are considered to be amongst the most prevalent foodborne pathogens associated with sporadic diarrhoea in humans (Engberg *et al*., 2001; Callicott *et al*., 2008; Horrocks *et al*., 2009; Chen *et al*., 2010). *Campylobacter* spp. colonise the intestines of food animals and they can contaminate meat during slaughter or post-slaughter processing (Hermans *et al*., 2011; Mackiw *et al*., 2012). Although *Campylobacter* infections are usually self-limiting and do not require antibiotic treatment, in some cases such as prolonged enteritis and septicaemia, antimicrobial treatment is needed. Macrolides and fluoroquinolones are the drugs of choice in treatment of human campylobacteriosis (Van Looveren *et al*., 2001; Guevremont *et al*., 2006; Moore *et al*., 2006), however emergence of resistance to these agents has prompted worries related to their use (Moore *et al*., 2005). In 2009, Zanoni and colleagues described a new *Campylobacter* species isolated from rabbit caecal contents named *C. cuniculorum*. So far there are no data on antimicrobial susceptibility in this novel *Campylobacter* species, so the aim of this study was to define for the first time the antimicrobial susceptibility in *C. cuniculorum* isolated from rabbits for meat.

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Materials and Methods

A selection of 29 *C. cuniculorum* isolates from a total of 29 epidemiologically non-correlated rabbits farms during a previous study by Revez *et al*. (2013) was used for this study: one strain for each farm was randomly selected for the evaluation of antibiotics susceptibility by using agar dilution method. The tested isolates were collected from April 2007 to November 2008 from 29 farms, 27 (18 intensive and 9 rural) were located in 7 different Italian regions while 2 farms (rural) were located in Portugal (Revez *et al*., 2013). The number of mares in the intensive farms ranged from 300 to 700 subjects; while in rural ones they ranged from 5 to 15. Information about the region and farm system is reported in Table 1. The minimum inhibitory concentration (MIC) value of ampicillin, ciprofloxacin, chloramphenicol, erythromycin, gentamicin, nalidixic acid, enrofloxacin and tetracycline was determined using a modified agar dilution method for *C. jejuni* and related species described by the Clinical and Laboratory Standards Institute (Wayne, PA, USA; CLSI, 2008) in order to be applied to the study of antimicrobial resistance of *C. cuniculorum*. The method was modified as follows: i) the base medium was Nutrient Broth N.2 (Oxoid, Basingstoke, UK) supplemented with 1.5% Bacto Agar (Difco-BD, Milan, Italy) and 5% defibrinated sheep blood; ii) the plates were incubated at 37°C±1 under microaerobic atmosphere with hydrogen for 72 h. These changes have been introduced because several isolates of *C. cuniculorum* did not grow on Mueller Hinton Agar with 5% defibrinated sheep blood; moreover, the reading times were increased since visible growth does not appear before 72 h of incubation for this species. All antimicrobial agents were purchased from Sigma-Aldrich (Saint Louis, MO, USA) and the antibiotic concentrations ranged from 0.015 to 128 μg mL^{-1}. *C. jejuni* ATCC 33560, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 29213 were used as a quality control strains. In this study the MICs resistance breakpoints for *Campylobacter* spp. were those used by the National Antimicrobial Resistance Monitoring System (Atlanta, GA, USA; NARMS) as reported in the US Centers for Disease Control NARMS Annual Report (2010) for *Campylobacter* spp. for chloramphenicol, ciprofloxacin, nalidixic acid and tetracycline. For ampicillin, erythromycin and gentamicin we adopted the breakpoints described by CLSI (2008) for *Campylobacter* spp. Since a standardised MIC breakpoint for enrofloxacin is not available for *Campylobacter* spp., we adopted the value indicated by CLSI (2008) for *Enterobacteriaceae*. The following resistance breakpoints were used: ampicillin≥32, chloramphenicol≥32, ciprofloxacin≥4, enrofloxacin≥4, erythromycin≥32, gentamicin≥8, nalidixic acid≥64, and tetracycline≥16.

Results

The results of MIC testing for each single isolate are reported in Table 1, while the distribution of MIC values of the 29 isolates tested is shown in Table 2. A monomodal distribution (with one distinct peak) for the MICs was found for all the antibiotics tested except for ciprofloxacin, enrofloxacin and erythromycin, which showed a bimodal (with two distinct peaks) appearance with a second peak at 4-128, 1-16 and 128-≥128 μg mL^{-1}, respectively. The bimodal distribution means that there are two distinct microbial populations, showing a different behaviour to antimicrobials, suggesting an acquired resistance. Noteworthy 15 and

13 isolates, all obtained from rabbits reared in intensive farms, showed a resistance to erythromycin and enrofloxacin characterised by a bimodal frequency with a high level of MIC in the second peak.

Discussion

This is the first report on the antimicrobial susceptibility of *C. cuniculorum*. Revez *et al*. (2013), investigating the occurrence of ε-proteobacteria in caecal contents of rabbits, isolated *C. cuniculorum* in 83 out of 87 animals tested, in a large number of colonies, suggesting that this microorganism, when present,

Table 1. Results of the minimum inhibitory concentration test of the twenty-nine *Campylobacter cuniculorum* isolates from rabbits with relative information on locality (region) and farm system.

Farm code	Region	Farm system	MIC values (g mL^{-1}) of 8 antimicrobials							
			CIP	NA	ENR	AMP	TE	GM	E	C
1	Emilia-Romagna	Rural	0.125	16	0.06	2	4	0.25	1	16
9	Emilia-Romagna	Rural	0.5	32	0.06	4	8	0.125	1	16
21	Beira litoral (PT)	Rural	0.125	32	0.125	4	8	0.06	1	16
22	Algarve (PT)	Rural	0.25	16	0.125	16	4	0.125	0.5	8
23	Emilia-Romagna	Rural	0.25	32	0.06	16	8	0.125	1	16
24	Emilia-Romagna	Rural	0.5	64	0.125	4	4	0.125	2	16
25	Emilia-Romagna	Rural	0.25	32	0.06	16	4	0.25	4	16
26	Emilia-Romagna	Rural	0.125	32	0.06	1	4	0.06	0.5	8
27	Lazio	Rural	0.25	32	0.125	8	4	0.125	1	16
28	Lazio	Rural	0.125	32	0.06	2	4	0.03	1	8
29	Lazio	Rural	0.125	32	0.06	2	4	0.03	1	8
2	Piemonte	Intensive	0.125	128	0.06	8	32	2	>128	8
3	Emilia-Romagna	Intensive	64	>128	16	2	32	4	>128	8
4	Emilia-Romagna	Intensive	0.25	128	0.06	16	64	8	>128	8
5	Emilia-Romagna	Intensive	32	>128	8	8	4	0.03	1	16
6	Veneto	Intensive	32	128	4	8	32	1	>128	16
7	Veneto	Intensive	32	>128	8	8	32	4	>128	8
8	Sicilia	Intensive	0.5	64	0.125	8	32	1	>128	16
10	Emilia-Romagna	Intensive	8	>128	2	8	16	0.03	>128	4
11	Emilia-Romagna	Intensive	64	>128	8	4	32	0.5	0.5	16
12	Friuli Venezia Giulia	Intensive	64	>128	8	8	32	1	>128	16
13	Veneto	Intensive	128	>128	16	8	32	4	>128	16
14	Veneto	Intensive	16	64	1	2	8	0.125	1	16
15	Marche	Intensive	4	128	4	0.5	16	2	>128	4
16	Veneto	Intensive	16	16	2	1	16	0.5	128	8
17	Veneto	Intensive	32	128	8	16	64	4	>128	8
18	Marche	Intensive	0.125	16	0.03	2	32	0.03	>128	2
19	Emilia-Romagna	Intensive	32	>128	4	2	64	0.5	>128	8
20	Lazio	Intensive	0.5	32	0.125	16	32	0.125	>128	8

MIC, minimum inhibitory concentration; PT, Portugal; CIP, ciprofloxacin; NA, nalidixic acid; ENR, enrofloxacin; AMP, ampicillin; TE, tetracycline; GM, gentamicin; E, erythromycin; C, chloramphenicol.

Table 2. Distribution of minimum inhibitory concentrations of twenty-nine *Campylobacter cuniculorum* isolates and minimum inhibitory concentration 50 and 90 values.

Antimicrobials	Number of *Campylobacter cuniculorum* isolates with MIC (μg mL^{-1})															
	≤0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128	MIC 50	MIC 90
Ampicillin						1	2	7	4	9	6					8	16
Chloramphenicol								1	2	12	14					8	16
Ciprofloxacin				7	5	4			1	1	2	5	3	1		0.5	64
Enrofloxacin		1	9	6			1	2	3	5	2					0.125	8
Erythromycin						3	9	1	1					1	14	64	>128
Gentamicin		5	2	7	2	3	3	2	4	1						0.25	4
Nalidixic acid											4	9	3	5	8	64	>128
Tetracycline									9	4	3	10	3			16	32

MIC, minimum inhibitory concentration.

colonises the caecum at a high concentration (Revez *et al*., 2013).

The results of this study, even if not statistically analysed, show high resistant level in *C. cuniculorum* isolated from rabbits reared in intensive farm; indeed, all the 18 isolates from intensive farms resulted resistant to two antibiotics at least. On the contrary, out of the 11 isolates from rural farms, only one resulted resistant to only one antibiotic. These data suggest that modern food animal production managements contribute to produce favourable conditions for the emergence and spread of antibiotic resistant bacteria due to the larger use of antimicrobial agents to control infections. Moreover, trends in antimicrobial resistance have shown a clear association between use of antibiotics in the veterinary industry and resistant isolates of *Campylobacter* spp. in humans (Alfredson and Korolick, 2007; Angulo *et al*., 2004).

Nowadays, there is no information on the pathogenic role of this new *Campylobacter* species, but the importance of antibiotic resistances that could be transmitted to other pathogen *Campylobacter* species may represent a risk of human concerns. Resistance to fluoroquinolones and macrolides is mediated by chromosomal mutations not transferable to other bacteria. However, the resistance to tetracycline show the potential for resistance transmission to other *Campylobacter* species (Aarestrup and Engberg, 2001).

In the present study, fluoroquinolone and macrolide showed a bimodal distribution suggesting an acquired resistance due to a gene mutation. Fluoroquinolones and macrolides are the antimicrobials chosen for the treatment of campylobacteriosis; in *Campylobacter* spp., fluoroquinolone resistance seems to be due to mutations in the *gyrA* gene encoding part of the GyrA subunit of DNA gyrase (Aarestrup and Engberg, 2001; Alfredson and Korolik, 2007). Relatively to macrolide resistance in *Campylobacter* species, modification of the target, represented by point mutation or methylation of 23S *rRNA* gene, seems to be the main mechanism involved. As far as *C. cuniculorum* is concerned, in-depth studies should be performed to clarify molecular mechanisms by sequencing the involved genes in *C. cuniculorum* isolates and performing comparisons of these sequences in sensible and resistant isolates. For all the other antibiotics tested, a monomodal distribution of MIC values was observed and, on the basis of the clinical breakpoints, we may assume that all *C. cuniculorum* isolates are sensitive to chloramphenicol and ampicillin. Sixteen out of the 29 tested isolates resulted resistant to nalidixic acid and tetracycline. Regarding the nalidixic acid, 12 out of 16 isolates resistant to nalidixic acid were resistant to ciprofloxacin too, so, the quinolone resistance-determining region of *gyrA* gene could be involved in the acquisition of this resistance. With regard to tetracycline resistance, it is found to be located in *C. jejuni* and *C. coli* on a self-transmissible plasmid encoding a ribosomal protection protein, designated as *tet (O)*, thus suggesting a potential role of *C. cuniculorum* in passing this resistance to other *Campylobacter* species.

Conclusions

In conclusion, for the first time this study shows data on *C. cuniculorum* antimicrobial susceptibility, suggesting a probable higher risk of antibiotic resistance in rabbits reared in intensive farms than those reared in rural farms. The evidence of tetracycline resistance in *C. cuniculorum* that could be transmitted to other human pathogen *Campylobacter* species may represent a risk for human health.

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