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.

**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 Revex 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 N2 (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.

**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; Guevermont 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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**Key words**: *Campylobacter cuniculorum*; Antibiotic resistance; Rabbits.

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romycin 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 *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 | CIP  | NA  | MIC values (µg mL⁻¹) of 8 antimicrobials |
|-----------|--------------|-------------|------|-----|---------------------------------------|
|           |              |             | 125  | 16  | ENR 0.06 AMP 2 TE 4 GM 0.125 E 1 C 16 |
| 1         | Emilia-Romagna| Rural       | 0.125| 16  | 0.06 2 4 0.125 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 0.125 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   | 0.25 | 128 | 0.06 16 2 32 4 128 8                |
| 4         | Emilia-Romagna| Intensive   | 0.25 | 128 | 0.06 16 64 8 128 8                 |
| 5         | Emilia-Romagna| Intensive   | 0.25 | 128 | 0.06 16 4 32 4 128 8                 |
| 6         | Veneto        | Intensive   | 0.25 | 128 | 0.06 16 4 32 4 128 8                 |
| 7         | Veneto        | Intensive   | 0.25 | 128 | 0.06 16 4 32 4 128 8                 |
| 8         | Sicilia       | Intensive   | 0.25 | 128 | 0.06 16 4 32 4 128 8                 |
| 10        | Emilia-Romagna | Intensive  | 0.25 | 128 | 0.06 16 4 32 4 128 8                 |
| 11        | Emilia-Romagna | Intensive  | 0.25 | 128 | 0.06 16 4 32 4 128 8                 |
| 12        | Friuli Venezia Giulia | Intensive | 0.25 | 128 | 0.06 16 4 32 4 128 8                 |
| 13        | Veneto        | Intensive   | 0.25 | 128 | 0.06 16 4 32 4 128 8                 |
| 14        | Veneto        | Intensive   | 0.25 | 128 | 0.06 16 4 32 4 128 8                 |
| 15        | Marche        | Intensive   | 0.25 | 128 | 0.06 16 4 32 4 128 8                 |
| 16        | Veneto        | Intensive   | 0.25 | 128 | 0.06 16 4 32 4 128 8                 |
| 17        | Veneto        | Intensive   | 0.25 | 128 | 0.06 16 4 32 4 128 8                 |
| 18        | Marche        | Intensive   | 0.25 | 128 | 0.06 16 4 32 4 128 8                 |
| 19        | Emilia-Romagna | Intensive  | 0.25 | 128 | 0.06 16 4 32 4 128 8                 |
| 20        | Lazio         | Intensive   | 0.25 | 128 | 0.06 16 4 32 4 128 8                 |

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

| Antimicrobials | ≤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 | 8 |   |    |     |    |    |    |    |      |       | >128  |
| Gentamicin     | 5      | 2    | 7    | 2     | 3    | 3   | 2 | 4 | 1 |    |     |0.25|4 |    |    |      |       |       |
| Nalidixic acid | 4      | 9    | 3    | 5     | 8    |    | 64 | 8 |   |    |     |    |    |    |    |      | >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 Korolik, 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 concern. 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 campylobacterioses; 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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