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1 **Antimicrobial resistance of *Enterococcus cecorum*: ECOFF determination**

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24 Key-words: *Enterococcus cecorum*, epidemiological cut-offs, disc diffusion, broth
25 microdilution, resistance genes, MICs

26 Running title: antimicrobial resistance of *Enterococcus cecorum*

27

28 **ABSTRACT**

29 *Enterococcus cecorum*, a commensal Gram-positive bacterium of the chicken gut, has
30 emerged as a worldwide cause of lameness in poultry, particularly in fast-growing broilers. It
31 is responsible for osteomyelitis, spondylitis and femoral head necrosis, causing animal
32 suffering, mortality and antimicrobial use. Research on the antimicrobial resistance of *E.*
33 *cecorum* clinical isolates in France is scarce, and epidemiological cut-off (ECOFF) values
34 unknown. To determine tentative ECOFF (CO_{WT}) values for *E. cecorum* and to investigate the
35 antimicrobial resistance patterns of isolates from mainly French broilers, we tested the
36 susceptibility of a collection of commensal and clinical isolates (n=208) to 29 antimicrobials
37 by the disc diffusion (DD) method. We also determined the minimum inhibitory
38 concentrations (MICs) of 23 antimicrobials by the broth micro-dilution method. To detect
39 chromosomal mutations conferring antimicrobial resistance, we investigated the genomes of
40 118 *E. cecorum* isolates mainly obtained from infectious sites and previously described in the
41 literature. We determined the CO_{WT} values for more than 20 antimicrobials and identified two
42 chromosomal mutations explaining fluoroquinolone resistance. The DD method appears
43 better suited for detecting *E. cecorum* antimicrobial resistance. Although tetracycline and
44 erythromycin resistances were persistent in clinical and non-clinical isolates, we found little
45 or no resistance to medically important antimicrobials.

46

47 **INTRODUCTION**

48 *Enterococcus cecorum*, first described as *Streptococcus cecorum* in 1983 (1), is a dominant
49 commensal Gram-positive bacterium found in the intestinal microbiota of healthy adult
50 chickens (1). Since 2002, it has been emerging in poultry production as a pathogen
51 responsible for lameness, osteomyelitis and spondylitis with increased mortality rates in
52 broiler productions and to a lesser extent in ducks and other avian species (2). The
53 development of *E. cecorum* infections is multifactorial, and depends on predisposing factors
54 related to host genetics, rapid growth, feed composition, husbandry procedures and animal
55 density, all in combination with the bacterium's pathogenic potential (3-5).

56 There are no vaccines against *E. cecorum*, and protective measures can only include
57 biosecurity and good poultry management. If an *E. cecorum* infection is diagnosed,
58 antimicrobials may be used as soon as possible to prevent further progression (4). Indeed,
59 antimicrobial therapy is ineffective for paralysed birds. The choice of the antimicrobial
60 compound to be used should be based on the susceptibility of the isolated strain. As defined
61 by EUCAST (<https://mic.eucast.org/>), epidemiological cut-off (ECOFF) values distinguish
62 microorganisms without (wild type) and with phenotypically detectable acquired resistance
63 mechanisms (non-wild type) to the agent in question, and a micro-organism is defined as wild
64 type (WT) for a species by the absence of acquired and mutational resistance mechanisms to
65 the drug in question. On the other hand, clinical breakpoints are determined on the basis of
66 dosages, pharmacokinetics, resistance mechanisms, MIC distributions, inhibition zone
67 diameters and more recently, pharmacodynamics and ECOFFs. A micro-organism is thus
68 defined as clinically susceptible by a level of antimicrobial activity associated with a high
69 likelihood of therapeutic success. Despite the few MIC criteria for *E. cecorum* proposed by
70 Borst et al. (6) for several antimicrobials, there are no ECOFFs nor clinical breakpoints

71 specifically for *E. cecorum*. Few studies on the antimicrobial resistance of *E. cecorum* are
72 available and interpretation is usually based on enterococci ECOFFs or breakpoints (6-9).

73 Resistance to tetracycline and erythromycin is the most commonly reported, but resistance to
74 aminoglycosides and β -lactams is sometimes detected (4, 9). To date, few acquired
75 antimicrobial resistance genes (9) and no resistance-conferring mutations have been
76 associated with antimicrobial resistance phenotypes in *E. cecorum*.

77 The aims of this study were to analyse by disc diffusion (DD) and broth micro-dilution
78 (BMD) methods a large collection of commensal and clinical *E. cecorum* isolates to: (i)
79 establish provisional ECOFF (CO_{WT}) values for *E. cecorum* inhibition zone diameters and
80 minimum inhibitory concentrations (MICs); (ii) to evaluate the correlation between inhibition
81 zone diameters and MIC values; and (iii) to analyse the relationship between observed
82 phenotypes and the genotypes of previously studied *E. cecorum* clinical isolates (J. Laurentie,
83 V. Loux, C. Hennequet-Antier, E. Chambellon, J. Deschamps, A. Trotureau, S. Furlan, C.
84 Darrigo, F. Kempf, J. Lao, M. Milhes, C. Roques, B. Quinquis, C. Vandecasteele, R. Boyer,
85 O. Bouchez, F. Repoila, J. Le Guennec, H. Chiapello, R. Briandet, E. Helloin, C. Schouler, I.
86 Kempf, and P. Serror, submitted for publication).

87

88 MATERIAL AND METHODS

89 Bacterial Isolates.

90 The present study investigated 208 isolates of *E. cecorum*. Of these, 118 were previously
91 described in the literature isolates (J. Laurentie, V. Loux, C. Hennequet-Antier, E.
92 Chambellon, J. Deschamps, A. Trotureau, S. Furlan, C. Darrigo, F. Kempf, J. Lao, M. Milhes,
93 C. Roques, B. Quinquis, C. Vandecasteele, R. Boyer, O. Bouchez, F. Repoila, J. Le Guennec,
94 H. Chiapello, R. Briandet, E. Helloin, C. Schouler, I. Kempf, and P. Serror, submitted for
95 publication), five were co-isolated with *E. coli* from an infected bird, and 85 were isolated

96 between 2019 and 2021 in France from the caecal contents or pooled faecal samples of
97 healthy birds collected from hatcheries, farms and slaughterhouses (**Table S1**). A selective
98 medium specific to *E. cecorum* was used to isolate commensal strains (S. Furlan & P. Serror,
99 personal communication).

100 **Disc diffusion method.** The DD method was applied to 29 antimicrobials or
101 associations of antimicrobials of importance in either veterinary or human medicine
102 (amoxicillin 25 µg, ampicillin 2 µg, cefotaxime 5 µg, ceftaroline 5 µg, imipenem 10 µg,
103 norfloxacin 10 µg, ciprofloxacin 5 µg, levofloxacin 5 µg, vancomycin 5 µg, teicoplanin 30 µg,
104 gentamicin 500 µg, streptomycin 300 µg, spectinomycin 100 µg, lincomycin-spectinomycin 2
105 µg/100 µg, lincomycin 15 µg, erythromycin 15 µg, spiramycin 100 µg, tylosin 30 µg,
106 quinupristin-dalfopristin 15 µg, tetracycline 30 µg, doxycycline 30 µg, tigecycline 15 µg,
107 tiamulin 30 µg, fosfomycin 200 µg, nitrofurantoin 100 µg, linezolid 10 µg, chloramphenicol
108 30 µg, bacitracin 130 µg and rifampicin 5 µg) on Mueller-Hinton agar plates supplemented
109 with 5% mechanically defibrinated horse blood and 20 mg/L β-NAD (MH-F agar). All the
110 procedures were in keeping with the EUCAST DD method (10). Plates were inoculated by
111 swabbing in three directions with a 0.5 McFarland bacterial suspension (5×10^7 colony
112 forming units (CFU)/mL), prepared in saline solution (0.85% NaCl). Inhibition zone
113 diameters were measured after 18 h ± 2 h of incubation (and 24 h more for glycopeptides) at
114 35°C ± 2°C in 5% CO₂, as recommended for *Streptococcus pneumoniae*. As there is no
115 recommended reference strain for *E. cecorum*, *S. pneumoniae* (CIP 104340) was used as a
116 proxy.

117 **Broth micro-dilution method.** The BMD method (11) was used to determine the
118 MICs of 23 antimicrobial agents for *E. cecorum* isolates placed in 96-well microtitre plates.
119 Firstly, 50 µL of sterile water was placed in each well, then 50 µL of the antimicrobial
120 solution was added to each well in the first column of the microtitre plate. Serial two-fold

121 dilutions of the antimicrobial solutions were performed by transferring 50 μ L from the first
122 column to the second one, and subsequently up to the lowest tested dilution as described in
123 **Figure 1**. The microtitre plates were stored at -70°C before use.

124 The day before the MIC determination assay, *E. cecorum* isolates were inoculated onto
125 Columbia agar supplemented with 5% of sheep blood from Bio-Rad and incubated at 35°C
126 for 24 hours in 5% CO_2 . Next, 0.5 McFarland bacterial suspensions prepared in saline
127 solution were diluted 1:100 in cation-adjusted Mueller-Hinton fastidious (MH-F) broth
128 (Thermo Scientific™) in order to reach the final concentration of 5×10^5 CFU/mL. Finally,
129 50 μ L of the suspension was added to each well. One well per plate was used as a positive
130 control (wells with only the bacterial suspension) and one as a negative control (wells with
131 only sterile cation-adjusted MH-F broth used to prepare the inoculum). The MICs were read
132 after 18 ± 2 h of incubation at $35 \pm 2^{\circ}\text{C}$ and 24 h more for streptomycin, as recommended for
133 enterococci. As there is no reference for *E. cecorum*, *S. pneumoniae* (CIP 104340) was used
134 as a proxy.

135 **Cut-off value determination and statistical tests.** For the DD method, the
136 normalised resistance interpretation (NRI) method was used with permission from the patent
137 holder, Bioscand AB, Täby, Sweden (European patent no. 1383913, US patent no. 7,465,559)
138 to determine the tentative cut-off value for each of the molecules tested. In the event of outlier
139 values, a maximum of 1% ($n=2$ isolates) of extremely high diameter values were removed for
140 analysis. For MICs, the ECOFFfinder tool
141 (https://www.eucast.org/mic_distributions_and_ecoffs/, v2.1) was used to determine the
142 tentative cut-off value for each molecule tested without removing any outliers. The temporary
143 cut-off considered was the ECOFF set at 99% of the estimated wild-type population. Isolates
144 resistant to at least three different antimicrobial families were considered multi-drug resistant
145 (MDR).

146 Scattergrams were plotted to compare the concordance between the DD and MIC
147 categorisation of isolates. The percentage of discrepancy (Pd) was calculated for each
148 antimicrobial agent tested by both methods. For a value less than 5%, the classifications
149 obtained with the two methods were considered as equivalent (**Table S2**).
150 Statistically significant differences in the distribution of wild-type (WT) and non-wild-type
151 (NWT) isolates of different origins (clinical poultry (CP) versus non-clinical poultry (NCP)
152 isolates) according to DD results were confirmed by the Chi² test or Fisher exact test for five
153 or fewer isolates, for a p-value less than 0.05

154 **Identification of gene mutations.** Mutations in genes that may be involved in
155 antimicrobial resistance were identified by multi-alignment of the corresponding proteins
156 using MultAlin (12). Only mutations specific to NWT isolates were selected.

157

158 **RESULTS**

159 **Determination of inhibition zone diameters.** The inhibition zone diameters were
160 determined for the 208 *E. cecorum* isolates using the DD method. For each DD assay, the
161 results obtained for the reference strain and the inoculation density complied with EUCAST
162 recommendations (data not shown).

163 The temporary cut-Off (CO_{WT}) value was determined for 25 molecules (**Figure 2, Table S3**).

164 The distribution of the diameters of four antimicrobials is shown in **Figure S1**, while WT and
165 NWT isolates are presented in **Figure 2**. The heterogeneity of diameters for four β -lactams
166 (ampicillin, amoxicillin, cefotaxime and ceftaroline) prevented the determination of CO_{WT}
167 values.

168 We tested several molecules for a few antimicrobial families: there was almost total
169 agreement between DD results obtained for ciprofloxacin and levofloxacin, with respectively
170 97.6% and 97.1% of WT isolates, whereas 74.5% were categorised as WT for norfloxacin.

171 The concordance between spiramycin and tylosin was 98.6% and yielded similar rates of
172 NWT isolates (48.1% to 47.6%). The classification according to DD for spiramycin and
173 tylosin was similar to that obtained for erythromycin for respectively 93.3% and 92.8% of
174 isolates. Most of the isolates tested using DD were resistant to tetracycline and doxycycline
175 (respectively 95.7% and 93.8%, with an agreement of 98%) while none showed resistance to
176 tigecycline.

177 Globally, there were no NWT isolates for imipenem and fewer than ten for ciprofloxacin
178 (n=5) and levofloxacin (n=6), teicoplanin (n=1), vancomycin (n=8), gentamicin (n=1),
179 streptomycin (n=5), fosfomycin (n=2), nitrofurantoin (n=2), linezolid (n=2), chloramphenicol
180 (n=1) and rifampicin (n=7). Thirteen percent of the isolates were NWT for tiamulin. Of all the
181 isolates, 43.3% were found to be MDR to up to eight different antimicrobial families,
182 including glycopeptides. Only three isolates (1.4%) were susceptible to all the molecules
183 tested.

184 Distributions of WT and NWT isolates according to their origin (**Table 1**) showed that
185 identical rates of CP and NCP isolates were NWT for tetracycline and doxycycline, as were
186 for bacitracin. There were significant differences in the ratios of WT and NWT isolates for
187 erythromycin, as there were respectively 65% and 40% of NWT for CP and NCP ($p=$
188 0.00038); likewise for norfloxacin, where the corresponding figures for NWT were 32% and
189 18% ($p= 0.025$). On the other hand, for spectinomycin and lincomycin-spectinomycin, 21% of
190 NCP isolates were NWT for both compared with 4% of CP isolates for spectinomycin
191 ($p=8.7 \times 10^{-5}$) and 8% for lincomycin-spectinomycin ($p= 6 \times 10^{-3}$).

192 **Determination of minimal inhibitory concentrations (MICs).**

193 For each MIC determination assay, the results obtained for the reference strain complied with
194 EUCAST recommendations (data not shown) (EUCAST, 2019). The distributions of the
195 MICs of 23 antimicrobials and their corresponding MIC₅₀ and MIC₉₀ values are displayed in

196 **Figure 1.** MIC values above the tested range were observed for seven antimicrobials
197 (vancomycin, teicoplanin, tylosin, tetracycline, doxycycline, bacitracin and avilamycin). For
198 glycopeptides, doxycycline and bacitracin, only one, two or three strains were concerned. The
199 greatest proportion of isolates with MIC values above the tested range were found for
200 tetracycline (n=128, 61.5%), tylosin (n=54, 26%) and avilamycin (n=41, 19.7%). For
201 teicoplanin, tetracycline and rifampicin, the MIC₅₀ and MIC₉₀ values were equal (respectively
202 0.125 mg/L, 256 mg/L and 0.125 mg/L). For ten (ampicillin, amoxicillin, vancomycin,
203 gentamicin, doxycycline, tigecycline, chloramphenicol, linezolid, avilamycin and
204 daptomycin) of the tested antimicrobials, the values between MIC₉₀ and MIC₅₀ differed by
205 only one dilution step.

206 CO_{WT} values were calculated for 17 antimicrobial agents for the complete *E. cecorum* dataset
207 (**Figure 2**). No value could be computed for beta-lactams nor for spectinomycin due to the
208 heterogeneity of the population distribution. For tetracycline, doxycycline and avilamycin,
209 CO_{WT} values were determined through a graphical representation due to the number of NWT
210 strains. For seven antimicrobial agents, the percentages of NWT isolates were high to
211 extremely high (tylosin (38%), spiramycin (38.5%), lincomycin (42.8%), erythromycin
212 (44.2%), tetracycline (77.9%), doxycycline (81.7%) and avilamycin (90.9), whatever their
213 origin.

214 **Comparison between MICs and inhibition zone diameters for *E. cecorum*. Table S2**
215 highlights the difference in interpretation between DD and BMD methods. For nine
216 antimicrobial agents, the Pd values were lower than 5% (levofloxacin 4.8%, vancomycin
217 3.85%, teicoplanin 0.5%, gentamicin 1%, quinupristin-dalfopristin (QDF) 2.9%, tigecycline
218 0.0%, chloramphenicol 0.5%, linezolid 1% and rifampicin 2.4%), meaning that there were
219 very few differences in WT/NWT categorisation between the methods. In contrast, nine
220 antimicrobial agents yielded Pd values higher than 5% (ciprofloxacin 7.7%, streptomycin

221 9.6%, lincomycin 24.5%, erythromycin 16.4%, spiramycin 14.4%, tylosin 15.4%,
222 doxycycline 14.9%, tetracycline 17.8%, and bacitracin 17.8%). For macrolides, lincosamides
223 and streptogramins (MLS), tetracyclines and bacitracin, CO_{WT} values determined by the DD
224 method gave more NWT strains than BMD did. Conversely, for ciprofloxacin and
225 streptomycin, CO_{WT} values determined by the BMD method gave more NWT strains than
226 DD.

227 **Consistent gene content and expression of resistance phenotypes.** In our previous genomic
228 study isolates (J. Laurentie, V. Loux, C. Hennequet-Antier, E. Chambellon, J. Deschamps, A.
229 Trotureau, S. Furlan, C. Darrigo, F. Kempf, J. Lao, M. Milhes, C. Roques, B. Quinquis, C.
230 Vandecasteele, R. Boyer, O. Bouchez, F. Repoila, J. Le Guennec, H. Chiapello, R. Briandet,
231 E. Helloin, C. Schouler, I. Kempf, and P. Serror, submitted for publication), antimicrobial
232 resistance genes were identified on the genomes of 118 isolates and correspondences between
233 their genomic content and their resistance phenotype are presented below (**Table S4**).

234 The 114 tetracycline-NWT isolates with sequenced genomes carried at least one tetracycline
235 resistance gene. The *tet(M) tet(L)* association was the most frequent (n=61), followed by
236 *tet(M)* gene only (n=47), the associated *tet(M) tet(O)* genes (n=2), *tet(M)*, *tet(L)* and *tet(O)*
237 (n=1), *tet(M)* and *tet(44)* (n=1), the *tet(O)* gene (n=1) and the *tet(L)* gene (n=1). Thus, the
238 ribosomal protection gene *tet(M)* is the most frequent tetracycline resistance gene (n=112) in
239 our *E. cecorum* isolates, as reported for *E. cecorum* (9) and other enterococci [16]. No
240 tetracycline-resistance gene was detected in the genomes available for the four tetracycline-
241 WT isolates. As expected, most of our *erm*-positive isolates were NWT for erythromycin
242 (78/80, 97.5%) and lincomycin (77/80, 96.3%). Four isolates bearing *mefA* and *lnuC* were
243 NWT for lincomycin but not the other tested MLSs. The four isolates possessing the *ermG*,
244 *mefA*, *msrD* and *lnuC* genes were NWT for erythromycin, but only one was also NWT for
245 lincomycin, spiramycin and tylosin. Six of the eight QDF-resistant isolates for which genomic

246 sequences were available carried the *ermB* or *ermG* gene, but no other acquired resistance
247 mechanisms could be detected in the remaining two. Two isolates with an *ermB* gene were
248 found WT for macrolides. These diverse discrepancies between MLS phenotypes and
249 genotypes were not investigated further, but could result from the poor or lack of expression
250 of the encoded proteins, and/or the presence of unknown resistance genes or mutations.
251 Acquired resistance genes were identified to explain resistance to gentamicin, streptomycin or
252 spectinomycin in six aminoglycoside-resistant isolates. The *spc* (or *ant(9)-Ia*) gene was
253 detected in two spectinomycin-resistant isolates. Four isolates contained *aadE* (*ant(6)-Ia*), an
254 aminoglycoside-nucleotidyltransferase conferring resistance to streptomycin, and were found
255 resistant to this antimicrobial according to both BMD and DD methods. Two isolates from the
256 United States have been reported to carry the *aph(3')-III* gene conferring resistance to
257 kanamycin (9), but this aminoglycoside was not tested. According to the DD or BMD method,
258 26.4% or 16.3% of isolates were NWT for bacitracin. Among the sequenced isolates, 27 were
259 found to be NWT using DD and 24 of these NWTs had a bacitracin resistance operon, with
260 *uppP2*, *bcrB*, *bcrA* and *bcrR* genes. This operon was also detected in only four of the 91
261 bacitracin-WT sequenced isolates. With regard to glycopeptides, seven isolates were
262 classified as NWT for vancomycin according to DD. One of them was also NWT for
263 teicoplanin and a *vanA* operon was detected in its genome. No other glycopeptide resistance
264 genes were detected.

265 For fluoroquinolones, we looked in the 118 available genome sequences for chromosomal
266 mutations that could explain resistance as they may target DNA gyrase and topoisomerase IV.
267 The three ciprofloxacin- and levofloxacin-NWT sequenced isolates harboured a mutation in
268 the GyrA quinolone resistance-determining region (either S83Y or S83V), only detected in
269 these genomes. Moreover, a mutation in ParC (S82I) was also highlighted in 24/35 of
270 norfloxacin-NWT isolates.

271

272 **DISCUSSION**

273 For the first time, the CO_{WT} values for 25 out of 29 and 20 out of 23 molecules tested using
274 the DD or BMD methods respectively were calculated with the EUCAST method adapted to
275 *E. cecorum*, (or in three cases by visual inspection for BMD) the main exception being for β -
276 lactams, which have a heterogeneous distribution. The availability of tentative ECOFF values
277 is the first step in monitoring the AMR of a bacterial species. Our data are based on results
278 from one laboratory only; however, they concern a variety of commensal and clinical isolates
279 collected over more than 37 years. They will no doubt contribute to the definition of official
280 interpretative criteria for *E. cecorum*, a major poultry pathogen. Data from other countries
281 relative to multiple and diverse sources and a large number of isolates are now needed to set
282 recognised ECOFFs with the expected precise estimates (13).

283 This work reveals that the concentration criteria currently used to classify *E. cecorum* isolates,
284 based on the breakpoints recommended for enterococci or ECOFF values for *E. faecalis* and
285 *E. faecium*, do not always properly distinguish WT from non-WT, while the ECOFF values
286 for *S. pneumoniae* coincide more frequently (**Table 2**). Part of this difference is probably
287 related to the similar growth conditions under CO₂ 5% for *E. cecorum* and *S. pneumoniae*.
288 Overall, discrepancies between the distribution of MICs and CO_{WT} or ECOFF values for *E.*
289 *cecorum* and *S. pneumoniae*, *E. faecium* or *E. faecalis* were observed for aminoglycosides,
290 tigecycline, avilamycin and bacitracin. Indeed, *E. cecorum* seems susceptible to moderate
291 concentrations of aminoglycosides, and the CO_{WT} determined for streptomycin (32 mg/L) and
292 gentamicin (4 mg/L) are lower than the ECOFF for *E. faecium* (respectively 128 mg/L and 32
293 mg/L) or *E. faecalis* (512 and 64 mg/L). Currently there are no ECOFF values for these
294 antimicrobials for *S. pneumoniae*. The calculated CO_{WT} for tigecycline is 4 mg/L, which is
295 much higher than the ECOFF for *S. pneumoniae* (0.125 mg/L) or *E. faecium* (0.25 mg/L).

296 Both the MIC₅₀ and MIC₉₀ for tigecycline are low (respectively 0.5 and 1 mg/L) and all 208
297 isolates were classified as WT according to both DD and BMD methods. Our MIC results for
298 avilamycin yielded a MIC₅₀ of 64 mg/L and a CO_{WT} value of 1 mg/mL compared to ECOFF
299 values of 8 mg/L or 16 mg/L respectively for *E. faecalis* or *E. faecium*, resulting in a
300 percentage of avilamycin-NWT *E. cecorum* of 91.0%. The calculated CO_{WT} for bacitracin was
301 also three double dilutions lower than the ECOFF value for *E. faecium*. The size of the NWT
302 population may also bias the determination of the CO_{WT}. We could not calculate the CO_{WT} of
303 beta-lactams for *E. cecorum*. The MIC₉₀ values for ampicillin and amoxicillin were lower
304 (0.25 mg/L and 0.125 mg/L respectively) than the MICs reported for *E. faecalis* or *E. faecium*
305 (<https://mic.eucast.org/>), a feature of *E. cecorum* already reported for penicillin by Jung et al.
306 (4). Using an amoxicillin breakpoint of 0.25 mg/L like Borst et al. (6) we managed to classify
307 nine out of 208 isolates as amoxicillin-resistant (4.0%) in contrast to the 26.3% NWT rate
308 reported by the authors. This observation suggests a low rate of amoxicillin resistance in our
309 collection.

310 A comparison of the interpretations between the DD and BMD methods used and the gene
311 content of the sequenced isolates shows that the DD method is more consistent with the gene
312 content. For erythromycin, for example, using the DD method, 82/84 NWTs have an *erm*-
313 gene and only two WT isolates carry one. Conversely, according to the BMD method, 13
314 isolates of the 47 considered as WT carry an *erm* gene and 66/71 of the NWTs carry one. The
315 same is true, to a lesser extent, for tetracycline: 114/114 NWTs classified by the DD method
316 carried tetracycline resistance genes while 110/114 are considered NWTs by the BMD
317 method and carry at least one *tet* gene. These different observations suggest that under the
318 conditions tested, the DD method and the corresponding calculated CO_{WT} value are more
319 accurate in determining the resistance of *E. cecorum*.

320 Despite a significant decrease in the use of antimicrobials in animals during the last ten years
321 in France, Europe and the USA, tetracyclines and penicillins are still used extensively (14).
322 The prevalence of NWT isolates for tetracycline was extremely high, as previously observed
323 for enterococci from French broilers (15). High rates have also been reported for *E. cecorum*
324 in other countries (70%) (4), as well as for *E. faecium* (80.3%) and *E. faecalis* (78.5%) (16).
325 The prevalence of NWT isolates for erythromycin, a 14-membered macrolide, was quite high
326 (44.7% for BMD and 54.8% for DD), although lower than in data previously reported for *E.*
327 *cecorum* (70% (4)), and similar to those reported for European broiler isolates of *E. faecium*
328 (57.0% %) and *E. faecalis* (56.6%) (16). CP isolates were more frequently resistant to MLSs
329 (erythromycin, spiramycin and tylosin) than NCP ones. This difference has already been
330 observed in other studies (6, 7, 9, 17). Indeed, macrolides are used for therapy for poultry,
331 which may explain the prevalence of resistance in clinical isolates. A slightly higher number
332 of *E. cecorum* strains were found to be NWT for lincomycin (60.6% according to DD), but
333 with a MIC₅₀ of 1 mg/L, this species does not seem naturally resistant to lincosamides, unlike
334 several other *Enterococcus* species (e.g. *E. faecalis*, *E. avium*, *E. gallinarum*, *E.*
335 *casseliflavus*). The association of lincomycin (66% of NWTs in CP isolates) and
336 spectinomycin (4% of NWTs in CP isolates) highlights the benefits of lincomycin, as only 8%
337 of CP isolates are NWT for lincomycin-spectinomycin. This susceptibility may explain the
338 reported use in Germany of the antimicrobial association during the first week of life to
339 control *E. cecorum* isolates (18). Similarly, the MIC₉₀ of quinopristin-dalfopristin was low (2
340 mg/L) for our *E. cecorum* isolates, and precludes an intrinsic resistance to these compounds,
341 unlike *E. faecalis*. Indeed, only 6.2% of isolates were classified as NWT for quinopristin-
342 dalfopristin. It should be remembered that high occurrences of quinopristin-dalfopristin-NWT
343 *E. faecium* were observed in poultry in France and other European countries several years
344 ago, probably due to the use of virginiamycin as a growth promoter in Europe up to 1999 (15,

345 19). Unfortunately, this quinopristin-dalfopristin resistance apparently persisted in *E. faecium*
346 up to recent observations, maybe as a result of co-selection by other antimicrobials used for
347 therapy (19). Different resistance genes to MLS (*ermB*, *ermG*, *lnuB*, *merfA*, *msrD*, *linB*, *lnuB*,
348 *lnuC* and *lnuD* have already been identified by Sharma et al. in *E. cecorum* genomes and
349 associated with resistance phenotypes (9).

350 Avilamycin is an oligosaccharide antimicrobial used as a growth promoter among poultry in
351 the European Union up to 2006. A link between the consumption of avilamycin and resistance
352 of *E. faecium* to this antimicrobial has been demonstrated in the literature (20, 21). In France,
353 the percentages of broilers or turkeys with avilamycin-resistant *E. faecium* were respectively
354 8.3% and 21.8% in 2007 (22). Thus the high level of resistance of *E. cecorum* (90.8% of
355 NWT, and a MIC₅₀ of 64 mg/L) to an antimicrobial that is no longer used is rather
356 unexpected, but we could not detect the previously described avilamycin resistance
357 mechanisms, i.e. the *emtA* gene or mutations in L16 (20).

358 Very few isolates were classified as NWT for chloramphenicol, fosfomycin and
359 nitrofurantoin. A low prevalence of chloramphenicol resistance has also been reported in
360 other studies for *E. cecorum* or *E. faecium* (4, 23). Since 1994, chloramphenicol is forbidden
361 in Europe for food-producing animals, but other phenicols (e.g. florfenicol) are authorised,
362 mainly for pigs and cattle, explaining more frequent chloramphenicol-NWT *E. faecalis* in
363 these productions (23). Fosfomycin has rarely been tested against poultry enterococci.
364 According to Schwaiger et al. (24) about 5% of *E. faecalis* from cloacal swabs of organically
365 and conventionally kept laying hens were resistant to fosfomycin. In enterococci, fosfomycin
366 can be related to mutations in the target enzyme MurA, or enzymatic modification of
367 fosfomycin, linked to the acquisition of transferable *fosB* genes or the high expression of the
368 *fosX* gene (25, 26) . Consistently with Suyemoto et al. (27) who detected no nitrofurantoin

369 resistance among 32 *E. cecorum* strains, the prevalence of this resistance in our collection is
370 below 1%.

371 Prevalence of *E. cecorum* resistance to critically important antimicrobials in human medicine
372 is quite low, confirming our prediction from genome analysis isolates (J. Laurentie, V. Loux,
373 C. Hennequet-Antier, E. Chambellon, J. Deschamps, A. Trotereau, S. Furlan, C. Darrigo, F.
374 Kempf, J. Lao, M. Milhes, C. Roques, B. Quinquis, C. Vandecasteele, R. Boyer, O. Bouchez,
375 F. Repoila, J. Le Guennec, H. Chiapello, R. Briandet, E. Helloin, C. Schouler, I. Kempf, and
376 P. Serror, submitted for publication). Low rates of linezolid NWT isolates agree with the low
377 detection levels among broilers in *E. faecium* and *E. faecalis* in 2012-2013 (15, 23). However,
378 recent studies on animal samples inoculated onto linezolid-supplemented media revealed that
379 linezolid-resistant enterococci (or staphylococci) may be frequently present although not
380 detected when analysing only strains from the dominant enterococci population isolated on
381 non-supplemented media (28). Linezolid has never been used in animal production, but
382 antimicrobial resistance mechanisms evidenced in animal isolates (e.g. *cfp*, *optrA* and *poxTA*
383 genes) could explain the co-selection of the described linezolid-resistant strains by the use of
384 other commonly used antimicrobials (e.g. tetracyclines, florfenicol in pigs) (29). Daptomycin
385 is not used in animal production either, but is an option for treating infections with multidrug
386 resistant or vancomycin-resistant enterococci (VRE) in human patients. In line with our very
387 low rate of daptomycin-NWT isolates, most isolates of *E. faecium* and *E. faecalis* and all *E.*
388 *hirae*, *E. durans* and *E. casseliflavus* isolates from healthy cattle, pigs and chickens in nine
389 EU countries yielded susceptible isolates (19). Likewise, no daptomycin-resistant *E. cecorum*
390 strain was present in the collection analysed by Suyemoto et al. in the USA (27). Tigecycline
391 is a glycolcycline developed to overcome tetracycline resistance mechanisms. The activity of
392 this last resort antimicrobial for human medicine appears to have been preserved up to now in
393 enterococci from animals, as the absence of tigecycline resistance in our *E. cecorum* isolates

394 is in line with the results described for *E. faecium*, *E. hirae*, *E. durans* and *E. casseliflavus*
395 isolates of animal origin (4). The low gentamicin resistance rates are also in line with the
396 findings reported for *E. faecium* and *E. faecalis* from animals in Europe (4) but higher levels
397 were sometimes observed for *E. cecorum* (6, 17). The prevalence of vancomycin resistance
398 was low, as observed in recent studies of *E. cecorum* (4). Noteworthy, the *vanA* operon has
399 already been detected in an *E. cecorum* isolate in Japan (30). Strikingly, although rifampicin
400 is not used in poultry production in France, seven isolates were classified as NWT. Resistance
401 to rifampicin in enterococci is usually associated with mutations in the RNA polymerase gene
402 (*rpoB*), but no mutations specific to *E. cecorum* NWT isolates were detected.

403 **Conclusion.** For the first time, the tentative cut-offs for 25 and 20 antimicrobials were
404 determined using respectively DD and BMD methods on a large collection of *E. cecorum*
405 with clinical and non-clinical origins. Further experiments could confirm or refine these cut-
406 off values. The DD method seems more appropriate to determine resistance thresholds for this
407 bacterium, as it is more in line with the genetic content. Resistance to medically important
408 antimicrobials (imipenem, vancomycin, gentamicin, tigecycline or linezolid) is rare. Mutated
409 GyrA (S83Y or S83V) and ParC (S82I) may explain resistance to some fluoroquinolones.
410 Moreover, this study revealed that while some resistances (tetracycline, erythromycin, etc.)
411 are still prevalent, strains of clinical origin showed significant levels of sensitivity for
412 antimicrobials authorised for poultry.

413

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427

428

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- 540
- 541

542 **Figure legends**

543 **Figure 1: MIC results and CO_{WT} determination.** White fields represent the range of
544 dilutions tested. MIC values equal to or lower than the lowest concentration tested are
545 presented as the lowest concentration. MIC values greater than the highest concentration
546 tested are presented as one dilution step above the test range. A black vertical line indicates
547 that the CO_{WT} value was calculated with the ECOFFinder tool. A dotted line indicates that the
548 CO_{WT} value was determined by visual inspection (not enough WT isolates to calculate it). No
549 CO_{WT} could be determined for ampicillin, amoxicillin and spectinomycin.

550 ^a Quinu-Dalfo: Quinupristin-Dalfopristin

551 **Figure 2: Heatmap of *E. cecorum* antimicrobial sensitivity determined by the disc**
552 **diffusion method.** Determination of WT (white), non-WT (black) and non-determined (grey)
553 status for each antimicrobial. NCP: non-clinical poultry isolate, CP: clinical poultry isolate,
554 CH: clinical human isolate. ND: CO_{WT} was not determined.

555 **Table 1:** Non-wild-type isolates according to their clinical origin.

556 **Table 2:** Comparison of *E. cecorum* CO_{WT} values with *S. pneumoniae*, *E. faecalis*, *E. faecium*
557 or criteria previously used for *E. cecorum*.

558 **Supplementary material**

559 **Table S1:** Isolates used in the study.

560 **Table S2:** Scattergram comparing MICs and inhibition zone diameters.

561 **Table S3:** CO_{WT} values, inhibition zone diameters and interpretations for the disc diffusion
562 method.

563 **Table S4:** Gene content, mutations and phenotype of resistance in 118 *E. cecorum* isolates.

564 **Figure S1:** Distribution of inhibition zone diameters and NRI method for CO_{WT}
565 determination. A. Quinupristin-Dalfopristin; B. Erythromycin; C. Vancomycin; D.

566 Amoxicillin. The orange dotted line represents the CO_{WT} value determined. No CO_{WT} could

567 be determined for amoxicillin.

568

Table 1: Non-wild-type isolates according to their clinical origin.

Antimicrobial	CP ^a (n=113)	NCP ^a (n=89)	P-value ^c
	No. NWT ^b (%)	No. NWT ^b (%)	
Norfloxacin	36 (32%)	16 (18%)	0.025
Ciprofloxacin	2 (2%)	3 (3%)	0.66
Levofloxacin	3 (3%)	3 (3%)	1
Vancomycin	7 (6%)	1 (1%)	0.08
Teicoplanin	1 (1%)	0 (0%)	1
Gentamicin	0 (0%)	1 (1%)	0.44
Streptomycin	2 (2%)	3 (3%)	0.66
Spectinomycin	4 (4%)	19 (21%)	8.19x10⁻⁵
Lincomycin/Spectinomycin	9 (8%)	19 (21%)	6x10⁻³
Lincomycin	75 (66%)	47 (53%)	0.05
Erythromycin	74 (65%)	36 (40%)	3.8x10⁻⁴
Spiramycin	67 (59%)	29 (33%)	1.6x10⁻⁴
Tylosin	66 (58%)	29 (33%)	2.6x10⁻⁴
Quinupristin/dalfopristin	7 (6%)	5 (6%)	0.86
Tetracycline	111 (98%)	83 (93%)	0.14
Doxycycline	108 (96%)	82 (92%)	0.3
Tigecycline	0 (0%)	0 (0%)	1
Tiamulin	9 (8%)	18 (20%)	0.01
Fosfomycin	1 (1%)	1 (1%)	1
Nitrofurantoin	1 (1%)	1 (1%)	1
Linezolid	2 (2%)	0 (0%)	1
Chloramphenicol	0 (0%)	1 (1%)	1
Bacitracin	26 (23%)	29 (33%)	0.12
Rifampicin	6 (5%)	1 (1%)	0.14

^a CP for clinical poultry and NCP for non-clinical poultry isolates

^b WT for wild-type isolates and NWT for non-wild-type isolates according to the disc diffusion method

^c P-value calculated with Chi² or Fisher exact test if n≤5. Significant p-values are in bold.

Table 2 – Comparison of *E. cecorum* CO_{WT} values with *S. pneumoniae*, *E. faecalis*, *E. faecium* ECOFF or criteria previously used for *E. cecorum*.

Antimicrobial	Broth micro-dilution MIC (mg/L)					Disc diffusion Inhibition zone diameter (mm)			
	CO _{WT} <i>E.</i> <i>cecorum</i>	ECOFF <i>S. pn</i> ^a	ECOFF <i>E.</i> <i>faecalis</i>	ECOFF <i>E.</i> <i>faecium</i>	Other criteria ^b	CO _{WT} <i>E.</i> <i>cecorum</i>	ECOFF <i>S.pn</i>	ECOFF <i>E.</i> <i>faecium</i>	ECOFF <i>E.</i> <i>faecalis</i>
Ampicillin	ND	0.06	4	8	0.25 ^d (AMX)	ND	(28)	10	10
Avilamycin	1		8	16					
Bacitracin	4		(32) ^f	32		22			
Chloramphenicol	8	8	32	32	16 ^e	22	21		
Ciprofloxacin	4	4	4	8	0.5 ^e (ENR ^g) 2 ^e	12	(18)		
Daptomycin	0.25	(0.5) ^f	4	8	4 ^e				
Doxycycline	1	0.5	0.5	0.5		24			
Erythromycin	0.5	0.25	4	4	0.5 ^d 4 ^e	21	22		
Fosfomycin			128			18			
Gentamicin	4		64	32	4 ^c 250 ^d	15		8	8
Imipenem		0.016	4	4		21		21	21
Levofloxacin	2	2	4	4		13	19		
Linezolid	8	4	ID	4	4 ^e	21	(22)	19	19
Nitrofurantoin			(32) ^f	256		21			15
Norfloxacin						18	12		12
Quinupristin- dalfopristin	4				2 ^e	20			
Rifampicin	1	(0.125) ^f				22	25		
Streptomycin	32		512	128	512 ^c 500 ^e	15		-	-
Teicoplanin	1	0.25	2	2		14	(18) ^f	(16) ^f	(16) ^f
Tetracycline	2	1	4	4	4 ^c 8 ^e	27	25		
Tigecycline	4	0.125	0.25	0.25	0.25 ^e	19			18
Tylosin	8				2 ^d 16 ^e	15			
Vancomycin	2	1	4	4	16 ^e	15	(16) ^f	12	12

^a *S. pn* : *Streptococcus pneumoniae*

^b Criteria used by Borst et al. (Borst et al., 2012) based on ^c breakpoints for enterococci (Clinical Laboratory Standards Institute, 2010) or ^d stratification using the overall median concentration into susceptible (below the median) and non-susceptible (above the median) or ^e criteria used by Jackson et al. (Jackson et al., 2015).

^f EUCAST tentative ECOFF

^g ENR for enrofloxacin

ECOFF values are obtained from the EUCAST website (mic.eucast.org/search/)

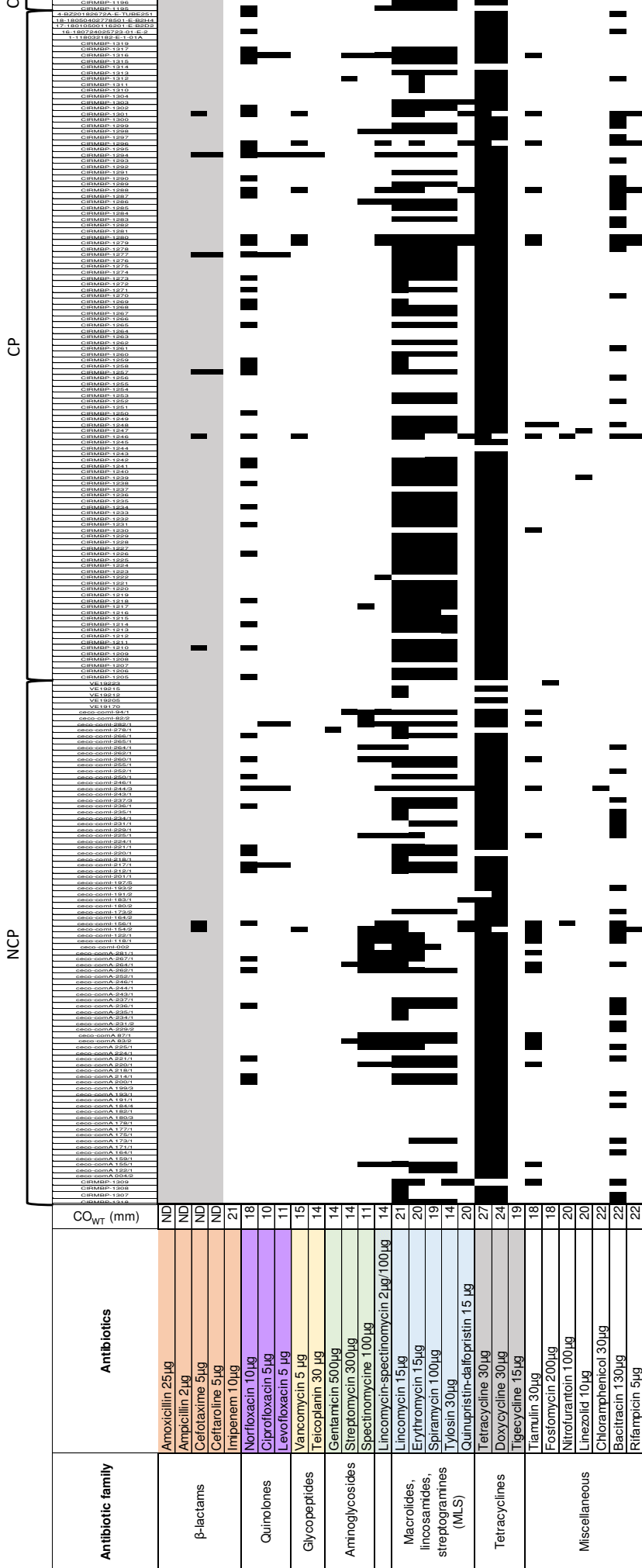


Figure 1: Heatmap of *E. cecorum* antimicrobial sensitivity determined by the disc diffusion method. Determination of WT (white), non-WT (black) and non-determined (grey) status for each antibiotic. NCP: non-clinical poultry isolate, CP: clinical poultry isolate, CH: clinical human isolate. ND: CO_{WT} was not determined

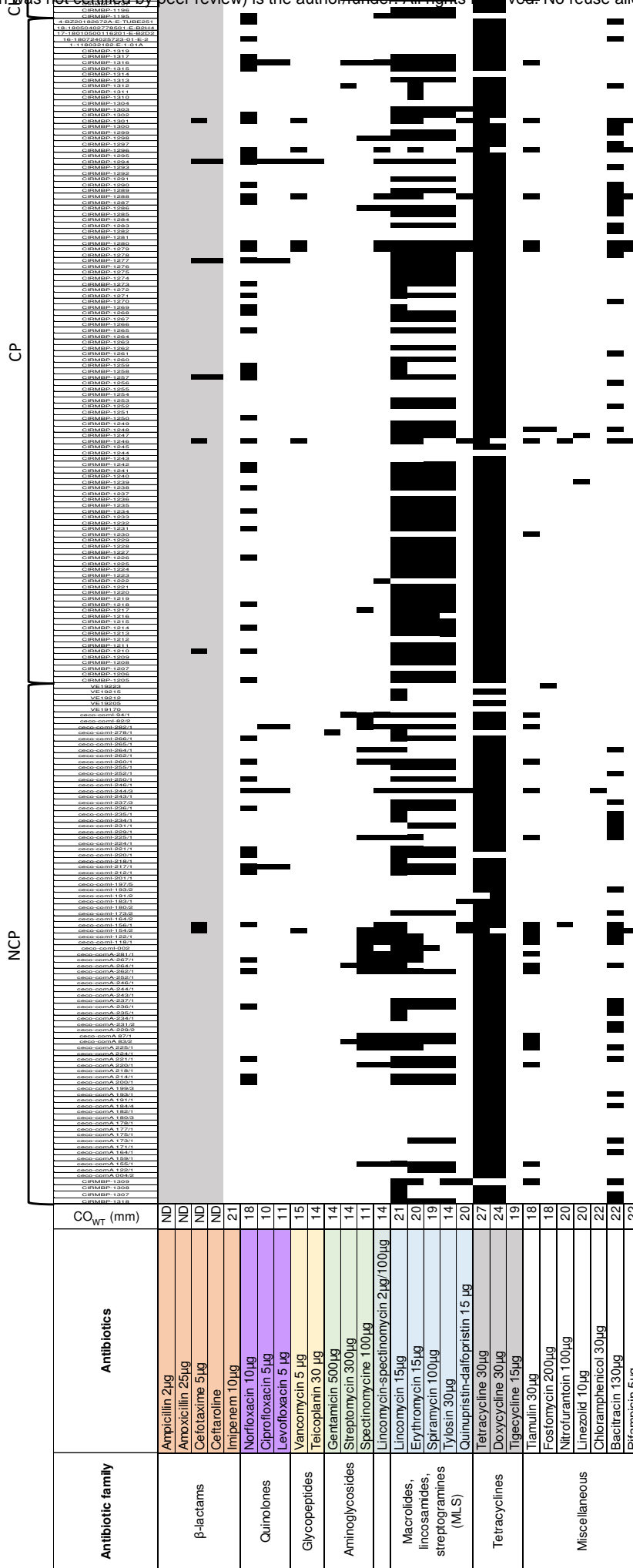


Figure 2: Heatmap of *E. cecorum* antimicrobial sensitivity determined by the disc diffusion method. Determination of WT (white), non-WT (black) and non-determined (grey) status for each antibiotic. NCP: non-clinical poultry isolate, CP: clinical poultry isolate, CH: clinical human isolate. ND: CO_{WT} was not determined