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Area of technical uncertainty for susceptibility testing of amoxicillin/clavulanate against Escherichia coli: analysis of automated system, Etest and disk diffusion methods compared to the broth microdilution reference

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1 **Title:**

2 **Area of technical uncertainty for susceptibility testing of amoxicillin/clavulanate against**
3 ***Escherichia coli*: analysis of automated system, Etest and disk diffusion methods compared**
4 **to the broth microdilution reference**

5

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18

19 **Abstract**

20 **Objectives:**

21 EUCAST recently warned about area of technical uncertainty (ATU) of amoxicillin/clavulanate
22 (AMX/C) disk susceptibility testing against *Enterobacterales*. Thus, we aimed to compare the
23 reliability of three routine methods and to evaluate the ATU impact.

24 **Methods:** 286 *Escherichia coli* strains (including 159 AMX-resistant) were categorized for the
25 two EUCAST AMX/C breakpoints by disk diffusion (Bio-Rad), Phoenix automate (Becton
26 Dickinson) and Etest (AES) compared to broth microdilution reference method.

27 **Results:** By microdilution, 84.2% of strains were AMX/C susceptible using urinary breakpoint
28 (MIC \leq 32 mg/L) and 62.2% using systemic breakpoint (MIC \leq 8 mg/L), with 63.6% of MICs
29 between 4 and 16 mg/L. For systemic breakpoint, category agreement (CA) and very major error
30 (VME) were unacceptable for Etest (71.7% and 27.3%), disk (73.1% and 23.4% at 19 mm cut-
31 off) and in a lesser extent for Phoenix (83.6% and 10.5%). For disks, unacceptable VME rate was
32 observed for diameters up to 22 mm, probably due to overcharged disks. For Etest, VME were
33 high at 6 mg/L (46/63) and 8 mg/L (22/29). For urinary breakpoint, CA was more acceptable for
34 disk (88.9%) and Etest (84.3%) (unevaluable for Phoenix).

35 **Conclusion:** AMX/C susceptibility testing of *E. coli* for systemic breakpoint was unreliable for
36 the three routine methods, mainly explained by the high prevalence (~60%) of strains with
37 microdilution MICs around the breakpoint (8 mg/L). Our data confirmed the EUCAST 19-20 mm
38 ATU for disk and suggested to introduce ATU for Etest MIC values of 6 and 8 mg/L.

39

40

41 INTRODUCTION

42 *Escherichia coli* remains one of the leading causes of bacterial infections in humans, mainly due
43 to its virulence for the urinary tract [1]. While amoxicillin (AMX) have represented for decades a
44 treatment of choice, half of *E. coli* strains worldwide are currently resistant to this antibiotic due
45 to β -lactamase production [2,3]. Thus, combinations with β -lactamase inhibitor such as
46 amoxicillin/clavulanate (AMX/C) have now a major role for the treatment of *E. coli* infections
47 [4,5].

48 Since 2014, EUCAST has distinguished two categorical breakpoints for susceptibility testing of
49 AMX/C against *Enterobacterales*, one for systemic infections (resistance for MIC >8 mg/L) and
50 one for uncomplicated urinary tract infections (resistance for MIC >32 mg/L). Since 2017,
51 EUCAST has implemented the concept of area of technical uncertainty (ATU) for pairs of
52 bacteria and antibiotic for which the interpretation of routine susceptibility testing is uncertain
53 [6]. For the *Enterobacterales* - AMX/C pair, disk susceptibility results are now considered
54 unreliable for the inhibition zone diameter of 19-20 mm using systemic breakpoint [7,8]. Due to
55 the relative paucity of data concerning this new ATU concept for the AMX/C - *E. coli* pair, we
56 aimed to analyze its potential impact on three different routine methods - disk diffusion, Phoenix
57 automated system and Etest - in comparison with broth microdilution (BMD) taken as reference.

58

59 MATERIALS AND METHODS

60 *Bacterial strains*

61 A set of 286 *E. coli* urinary strains were prospectively and consecutively collected for three
62 months from 102 outpatients and 184 inpatients in our 2500-bed hospital. Patients presented the
63 whole panel of urinary tract infections, from uncomplicated cystitis to severe sepsis requiring
64 intensive cares. Duplicate specimens defined as the same species from the same patient during

65 the period study were excluded. According to French regulation, no ethics committee approbation
66 was requested for this study. After being tested by automated Phoenix system as part of routine
67 work, strains were frozen at -80°C until further analysis.

68

69 *Amoxicillin-clavulanate susceptibility testing and categorization*

70 MIC were determined by BMD as reference method using Mueller–Hinton (MH) II broth (BD
71 Difco, France) in microtiter plates of 96 wells containing seven two-fold dilutions of AMX (1 to
72 64 mg/L) in the presence of potassium salt C concentrations of 2 mg/L (powders purchased from
73 Sigma-Aldrich, France). A 0.5 McFarland (McF) standard suspension monitored by a
74 photometric device was prepared for each strain, then diluted to obtain a final bacterial
75 suspension density of 5×10^5 CFU/mL. Plates were incubated at 35°C for 18 h before visual
76 assessment of turbidity. A growth control was used for each strain and results were considered as
77 valid when sufficient growth was observed, as obvious button or definite turbidity in a well
78 without antibiotic [9,10]. Each series ran 10 clinical strains as well as reference strain(s) for
79 quality control (QC): *E. coli* ATCC 25922 (MIC target: 4; range: 2-8 mg/L) for all strains and *E.*
80 *coli* ATCC 35218 producing TEM-1 β -lactamase (MIC target: 8-16; range: 4-32 mg/L) for
81 AMX-resistant strains. Automated susceptibility testing was performed using the UMIC/ID-97
82 panel of the Phoenix system (Becton Dickinson, Le Pont-de-Claix, France) according to
83 manufacturer's recommendations. Disk susceptibility testing was performed according to
84 EUCAST 2018 guidelines [11] by applying a 0.5 McF bacterial suspension and disks containing
85 20 μ g of AMX and 10 μ g of C (Bio-Rad, Marnes-la-Coquette, France) to the surface of a MH
86 agar plate (Bio-Rad, Marnes-la-Coquette, France). Plates were incubated for 18 h at 35°C prior to
87 determination of results. For Etest method, AMX/C strips from AES (Combourg, France) were

88 placed on the surface of a MH agar plate that was inoculated with a 0.5 McF bacterial suspension
89 like that used for the disk diffusion test, according to manufacturer's recommendations. The MIC
90 was determined by the intersection of the lower part of the ellipse shaped growth inhibition area
91 with the test strip. MIC values were reported as is without rounding to the next higher standard
92 dilution.

93 QC tests were performed for the routine methods using *E. coli* ATCC 25922 and *E. coli* ATCC
94 35218 according recommendations [11,12].

95 Susceptibility categorization was determined according to the two EUCAST criteria. For the
96 uncomplicated urinary tract infection breakpoint (below called urinary breakpoint), strains were
97 considered susceptible in case of AMX/C MIC values ≤ 32 mg/L or inhibition zone diameter ≥ 16
98 mm. For the other infections breakpoint (below called systemic breakpoint), strains were
99 considered susceptible in case of AMX/C MIC values ≤ 8 mg/L or inhibition zone diameter ≥ 19
100 mm (*i.e.*, the inhibition zone diameter recommended by EUCAST from 2014 to 2018). Moreover,
101 the percentage of susceptible isolates was calculated in the hypothesis of an inhibition diameter
102 cut-off of 20, 21 or 22 mm. As UMIC/ID-97 panel of Phoenix automated system provided
103 AMX/C MIC ranging from ≤ 2 mg/L to >8 mg/L, such method allowed categorization only
104 according to the systemic infection breakpoint.

105

106 *Data analysis*

107 Discrepancies of results were analyzed between a given routine method and the reference.
108 Coefficients of agreement were calculated both for the total set and the sub-group of AMX
109 resistant strains. Discrepancies were classified as follows: a major error (ME) when the routine
110 method classified the strain as resistant and the reference method as susceptible; a very major
111 error (VME) when the routine method classified the strain as susceptible and the reference

112 method as resistant. Rates of category agreement (CA) and discrepancies in susceptibility
113 categorization were compared between methods using Fisher's exact test. A p value <0.05 was
114 considered to be significant. For Etest, essential agreement (EA) was calculated using BMD as a
115 reference method according to standard definitions (*i.e.*, percentage of isolates with MIC within \pm
116 $1 \log_2$ dilution of the reference method MIC [13]).

117 Receiver operating characteristics (ROC) curves (<http://forge.info.univ-angers.fr>) were used to
118 evaluate agreement between disk diffusion and BMD reference.

119

120 **RESULTS**

121 *Broth microdilution method as reference method*

122 The AMX/C MIC values for QC strains were always within the range and most often on the
123 target (details in supplementary data [Table S1a]). Figure 1 shows the distribution of AMX/C
124 MICs for the total set according to their susceptibility or resistance to AMX. All the 129 AMX-
125 susceptible strains were fully susceptible to AMX/C (MIC₅₀ and MIC₉₀ of 4 mg/L). In contrast,
126 AMX/C MIC values were ≥ 2 mg/L for all the 157 AMX-resistant strains with MIC₅₀ of 16 mg/L
127 and MIC₉₀ ≥ 64 mg/L.

128

129 *Comparison of routine methods with reference method*

130 The MIC values and inhibition zone diameters determined by routine methods for QC strains are
131 reported in supplementary Table S1b-d. As AMX/C MIC values, the AMX/C inhibition zone
132 diameters were within the range for the two QC strains. Nevertheless, a median value of 2 mm
133 above the target value was noticed for the QC strain *E. coli* ATCC 25922 (Table S1d).

134 Tables 1 and 2 show the rates of susceptibility to AMX/C and the rates of concordance of the
135 routine methods compared to BMD according to urinary breakpoint (Table 1) or systemic

136 breakpoint (Table 2). All AMX-susceptible strains were correctly categorized as susceptible to
137 AMX/C whatever the method used. In contrast, marked differences were observed in the rate of
138 AMX/C susceptibility between methods for AMX-resistant strains, particularly for the systemic
139 breakpoint. Phoenix automated method seemed more reliable than disk diffusion and Etest with
140 an overall rate of susceptibility close to that of reference method. However, this result was
141 tempered by a total of 16.4% of errors (VME+ME) for the total set of strains (Table 2).

142 There was a poor correlation between AMX/C MICs determined by Etest and by BMD (Figure 2,
143 Table S2b), with up to 27.3% of VME using the systemic breakpoint (Table 2). Agreement was
144 acceptable for Etest values ≤ 4 mg/L (only 10 VMEs for the 161 isolates concerned) or ≥ 12 mg/L
145 (3 MEs among the 33 values). In contrast, categorization for Etest values of 6 and 8 mg/L was
146 unacceptable with 73.0% (46/63) of VMEs at 6 mg/L and 75.9% (22/29) at 8 mg/L, so the overall
147 EA (74.8%) was poor. Of note, 43 of these 78 VMEs correspond to isolates with a BMD MIC of
148 16 mg/L (Table S2b).

149 Disk diffusion also overestimated the AMX/C susceptibility of the AMX-resistant strains, but in
150 a lesser extent than Etest (Tables 1 and 2). For systemic breakpoint applied to AMX-resistant
151 strains, agreement was poor with the classical 19 mm diameter breakpoint: 67.5% of strains
152 estimated susceptible versus 31.2% by BMD, with 42.7% of VMEs (Table 2).

153
154 *Area of technical uncertainty*

155 Figure 3 shows the distribution of AMX/C inhibition zone diameters according to the AMX/C
156 MICs determined by BMD for the total set. The AMX/C inhibition zone diameters were all large
157 (from 22 mm to 31 mm) for the AMX-susceptible strains and more dispersed (from 10 mm to 26
158 mm) for AMX-resistant isolates. It was thus difficult to determine the best cut-off diameter. For
159 systemic breakpoint, it appeared that ATU of 19-20 mm proposed by EUCAST was appropriate

160 as 22 VME were observed for the 23 strains with such inhibition zone diameter. The use of 21 or
161 22 mm diameters as cut-off would allow to decrease VME ($p < 0.05$) and to improve CA for the
162 total set ($p < 0.05$) (Table 2). Besides, ME would increase if 22 mm cut-off was used instead of 21
163 mm. ROC curve analysis using 8 mg/L breakpoint indicated that diameter ≥ 22 mm would offer a
164 better compromise between specificity (81%) and sensitivity (69%).

165 On the other hand, Etest MIC values of 6 and 8 mg/L also represented a kind of ATU: reporting
166 these susceptibility results as resistant would decrease the VME rate from 27.3% to 3.8% for the
167 total set and improve CA from 71.7% to 86.7%.

168

169 **DISCUSSION**

170 This study underlined the difficulty in the assessment of AMX/C susceptibility testing against *E.*
171 *coli* by routine methods and the importance of the concept of ATU recently introduced by
172 EUCAST for *E. coli*-AMX/C pair [7].

173 Our results not only confirmed the 19-20 mm ATU for disk diffusion method, as pointed by the
174 2019 EUCAST warning for the breakpoint of systemic infections, but also highlighted the lack of
175 reliability of Etest for the 6 and 8 mg/L MIC values. This problem cannot be solved by repeating
176 the test (this was done for our set, with overall similar results; details not shown). Thus, the 6 and
177 8 mg/L Etest values might require an ATU warning for which it would be recommended to
178 perform another antimicrobial susceptibility method or to report the result as uncertain or
179 resistant in case of systemic infection.

180 Phoenix automated system yielded results closer to those obtained by BMD compared to
181 diffusion methods with two limitations: (i) about 10% of VME with the systemic breakpoint and
182 (ii) an inability to categorize numerous strains according to urinary breakpoint due to an

183 inappropriate range of AMX/C concentrations. Of note, this latter limitation of the Phoenix
184 system is also observed for automated systems of other manufacturers.

185 Whatever the routine method used in our study, the error rates were not acceptable according to
186 the FDA criteria (ME and VME rates >3%) [13]. As described by Humpries *et al.* [13], there
187 might be “a directly proportional relationship between the categorical error rate and the
188 percentage of isolates that hover around [...] the susceptible/resistant breakpoint if no
189 intermediate category is recognized” as for AMX/C - *Enterobacterales* pair in the recent
190 EUCAST recommendations [8]. Indeed, in our series, AMX/C MICs of around 6 out of 10 strains
191 (182 of 286) were within 1 log₂ dilution of the critical systemic breakpoint (8 mg/L). Of note, this
192 ratio is exactly in the range of that reported on the EUCAST website (6 091 out of the 10 159
193 strains collected in the ECOFF application - *i.e.*, 60.0% - had an MIC of 4, 8 or 16 mg/L). Thus,
194 the high error rates of routine methods for susceptibility testing of AMX/C against *E. coli* were
195 certainly favored by the large proportion of isolates with MICs around the systemic breakpoint.

196 Several previous series have underlined the difficulties to routinely evaluate the activity of
197 combinations of β -lactam and β -lactamase inhibitor against β -lactamase producing bacteria [14–
198 16]. For the systemic breakpoint, more than one strain out of five of our total set were categorized
199 as susceptible by disk and Etest but as resistant by BMD reference, the errors occurring
200 commonly for the same strains (CA of 92% between disk diffusion and Etest, $p>0.05$). Overall,
201 our results illustrated the poor correlation between methods performed in solid medium and those
202 performed in liquid medium potentially because of a better detection of β -lactamase expression in
203 liquid medium [15].

204 Such unreliability is of great concern, both at an individual and collective level as Etest is widely
205 used in routine given its easy implementation to determine AMX/C MIC in case of severe
206 infection although the reference method is BMD.

207 Given the differences in categorization observed, our study underlines how epidemiological data
208 could be biased by the method used. This might explain erratic variations over decades of the
209 resistance rates of *Enterobacteriales* to AMX/C described in many countries which markedly
210 contrast with stability or only long-term evolution for other antibiotic classes [17–19]. Though
211 isolates were from a single institution, our bacterial set was representative of *E. coli* strains
212 currently encountered in France as well as in some other European countries in terms of beta-
213 lactam resistance with around half strains resistant to AMX and 5% of extended-spectrum β -
214 lactamase producers [2]. Another strength of this study was the analysis of strains associated with
215 infections that vary greatly in severity: from cystitis for which the breakpoint of 32 mg/L is used
216 to pyelonephritis and prostatitis with bacteraemia for which the breakpoint at 8 mg/L has to be
217 applied. Thus, this bacterial set allowed to explore the diversity of *E. coli* infections encountered
218 routinely.

219 One limitation of this work could be the lack of molecular strain typing. However, clonality of
220 isolates can be reasonably ruled out both at individual level (exclusion of duplicate isolates) and
221 between patients (no epidemiological link between patients coming from a large geographical
222 area). Other limitations include the collection of isolates from a single hospital, the tests
223 performed by a single laboratory and the two-year delay between this collection and the last
224 susceptibility tests but loss of β -lactamase gene was avoided by freezing as shown by BMD tests
225 performed in duplicate with similar results between the beginning and the end of the procedure.
226 The major weakness was the evaluation of reagents and media from a single manufacturer by
227 system due to cost and time concerns, while variability from one company to another is
228 established [20,21]. The VME rates for disk diffusion method were too high not only for the 19-
229 20 mm EUCAST ATU zone but also for the 21 and 22 mm diameters. Even if the inhibition zone
230 diameters for the QC strains were always within the ranges recommended by EUCAST, these

231 diameters tended to be slightly on the large side for the wild-type QC strain *E. coli* ATCC 25922.
232 Recently, Åhman *et al.* [20] reported that Bio-Rad disks (those used in this study) tended to
233 overestimate the diameter inhibition zone with a mean value >2 mm from target value for the QC
234 strain *E. coli* ATCC 25922 (but still in the QC range). If we consider that our results for the 21
235 and 22 mm diameters were slightly overestimated by up to 2 mm and analyze our results
236 according to the ATU of 19-20 mm defined by EUCAST, the VME rate becomes then more
237 acceptable (5.2%, 15/286, Table S2). Thus, the very high VME rate for disk diffusion method
238 was probably due in part to the brand of the disks used at the time of the study. Of note, this
239 company then improved the quality of its discs [20].

240 In conclusion, AMX/C susceptibility testing of *E. coli* for systemic breakpoint appeared
241 unreliable for the three routine methods here tested. While the EUCAST 19-20 mm ATU for disk
242 diffusion was confirmed, our results also suggested to introduce an ATU for Etest AMX/C MIC
243 values of 6 and 8 mg/L. Our conclusions might not necessarily apply to other brands of media,
244 disks, or strips but caution is advised because the unreliability observed was mainly related to a
245 high proportion (60%) of strains with MIC around the systemic breakpoint as elsewhere in
246 Europe. Such difficulties in routine evaluation of AMX/C susceptibility are of great concern,
247 particularly for severe infections.

248

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251

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253 None to declare.

254

255 **Authors contributions:** FC, SB and MPC designed the study. AS, FLR and GB performed the
256 experiments. AS, SB, MPC and FC analyzed the data. AS and FC wrote the manuscript assisted
257 by SB and MPC. All authors amended and approved the final version of the manuscript.

258

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323

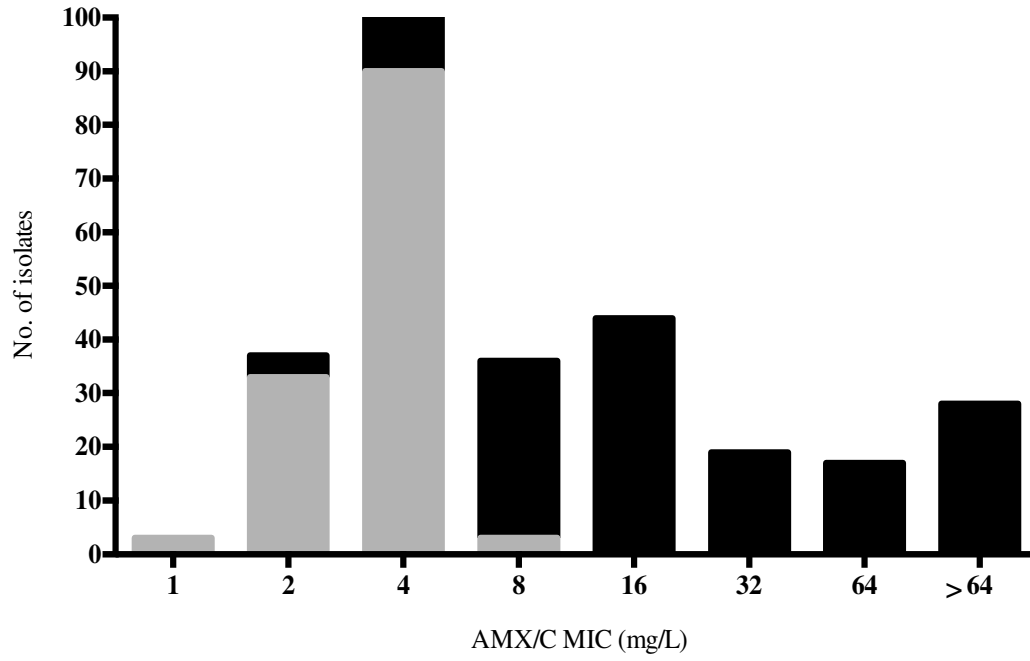


Figure 1

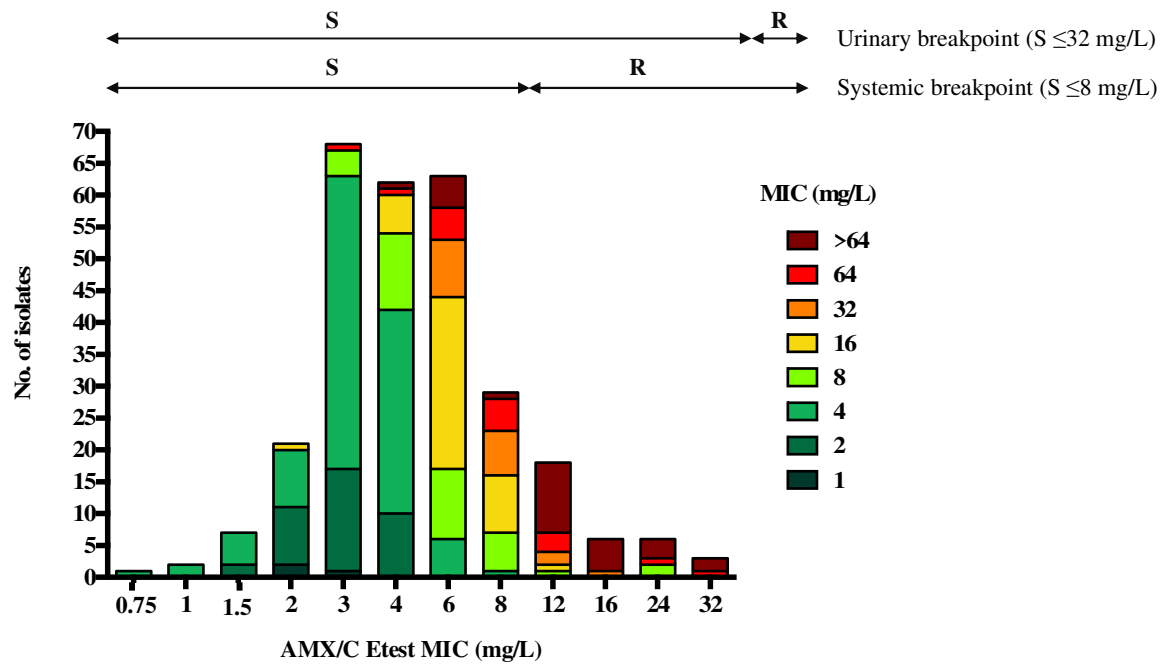


Figure 2

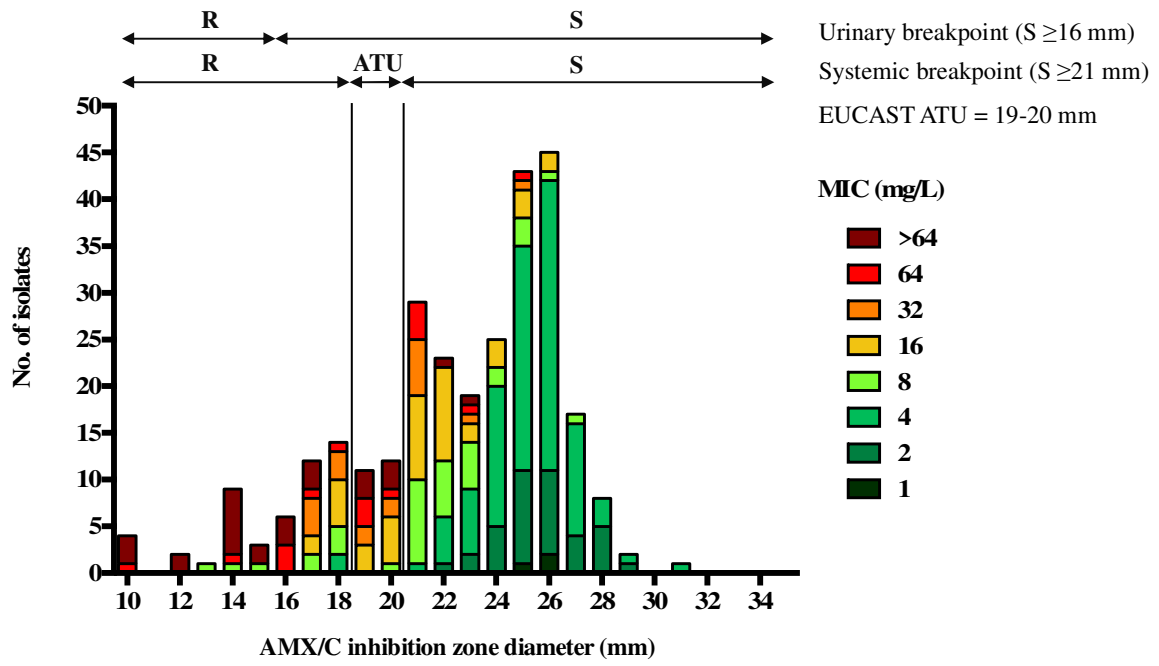


Figure 3

Table 1: Amoxicillin/clavulanate (AMX/C) susceptibility categorization and performances of disk diffusion (Bio-Rad) and Etest (AES) compared to broth microdilution as reference according to EUCAST urinary breakpoint for a set of 286 *Escherichia coli* strains among which 157 AMX-resistant strains.

Method	Total set (n = 286)				AMX resistant strains (n = 157)			
	AMX/C susceptibility (%)	Category agreement (%)	Very major error (%)	Major error (%)	AMX/C susceptibility (%)	Category agreement (%)	Very major error (%)	Major error (%)
Broth Microdilution	84.2	–	–	–	71.3	–	–	–
Etest	100 ^a	84.3	15.7	0	100 ^a	71.3	28.7 ^c	0
Disk	93.4 ^b	88.9	10.1	1.0	87.9 ^b	79.6	18.5 ^c	1.9

^a $p < 0.05$ versus broth microdilution and disk (two-by-two comparisons).

^b $p < 0.05$ versus broth microdilution and Etest.

^c $p < 0.05$ between disk and Etest.

Table 2: Amoxicillin/clavulanate (AMX/C) susceptibility categorization and performances of Phoenix automate (Becton Dickinson), disk diffusion (Bio-Rad) and Etest (AES) compared to broth microdilution as reference according to EUCAST systemic breakpoint for a set of 286 *Escherichia coli* strains among which 157 AMX-resistant strains. For disk, evaluation of 4 inhibition diameters due to technical uncertainty.

Method	Total set (n = 286)				AMX resistant strains (n = 157)			
	AMX/C susceptibility (%)	Category agreement (%)	Very major error (%)	Major error (%)	AMX/C susceptibility (%)	Category agreement (%)	Very major error (%)	Major error (%)
Broth microdilution	62.2	–	–	–	31.2	–	–	–
Phoenix	66.8 ^a	83.6 ^d	10.5 ^d	5.9	39.5 ^a	70.1 ^d	19.1 ^d	10.8
Etest	88.5 ^b	71.7	27.3	1.0	79.0 ^b	48.4	49.7	1.9
Disk								
≥19 mm	82.2 ^c	73.1	23.4	3.5	67.5 ^c	50.9	42.7	6.4
≥20 mm	78.3	76.9	19.6	3.5	60.5	57.9	35.7	6.4
≥21 mm	74.1	80.5	15.7	3.8	52.9	64.3	28.7	7.0
≥22 mm	64.0	83.6	9.1	7.3	34.4	70.0	16.6	13.4

^a $p < 0.05$ versus disk (for inhibition zone diameter ≥ 19 mm) and Etest (two-by-two comparisons).

^b $p < 0.05$ versus broth microdilution, Phoenix and disk (for inhibition zone diameter ≥ 19 mm).

^c $p < 0.05$ versus broth microdilution, Phoenix and Etest.

^d $p < 0.05$ versus disk (for inhibition zone diameter ≥ 19 mm) and Etest.