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A comparison of *E. coli* susceptibility for amoxicillin/clavulanic acid according to EUCAST and CLSI guidelines

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Purpose

In our tertiary care center, the reported susceptibility of *E. coli* blood isolates to amoxicillin/clavulanic acid exceeded 90% in 2005 and showed a progressive decrease to 50% by 2017. In this study, we investigate whether there is a real increase in resistant *E. coli* strains or if this apparent decline in reported susceptibility might be attributed to the substitution of CLSI by EUCAST guidelines in 2014.

Methods

We randomly selected 237 *E. coli* blood isolates (stored at -80°C) from 1985 to 2018 and reassessed their MIC-values, applying both the CLSI (fixed ratio of clavulanic acid) and EUCAST guidelines (fixed concentration of clavulanic acid). In parallel, the susceptibility of these isolates was retested by disk diffusion, according to the EUCAST guidelines. Whole genome sequencing was successfully performed on 233 of the 237 isolates.

Results

In only 130 of the 237 isolates (55.0%), testing according to the EUCAST and CLSI criteria delivered identical MIC-values for amoxicillin/clavulanic acid. In 64 of the 237 isolates (27.0%) the MIC-values diverged one dilution, in 38 (16.0%) two dilutions and in five (2.1%) three dilutions. From these 107 discrepant results, testing according to EUCAST methodology revealed more resistant profiles in 93 *E. coli* strains (94.1%). Also phenotypical susceptibility testing according to EUCAST guidelines tends to correlate better with the presence of beta-lactamase genes compared to CLSI testing procedure.

Conclusion

This study highlights the low agreement between EUCAST and CLSI methodologies when performing MIC-testing of amoxicillin/clavulanic acid. More strains are categorized as resistant when EUCAST guidelines are applied. The low agreement between EUCAST and CLSI was confirmed by WGS, since most of EUCAST resistant / CLSI sensitive isolates harbored beta-lactamase genes.

Keywords *Escherichia coli*, EUCAST, CLSI, Disk diffusion, Minimum inhibitory concentration, Resistance, Whole Genome Sequencing, Beta-lactamase

1. Introduction

Escherichia coli (*E. coli*) is the most common Gram-negative, rod shaped, facultative anaerobic bacterium in the human gastrointestinal tract [1]. It is the most frequently isolated Gram-negative micro-organism from adults suffering from bacteremia [2]. A common infectious focus for bacteremia is the urinary tract, which is also the most common extra-intestinal site colonized by these bacteria [3]. Gut translocation or the passage of bacteria across the mucosal barriers to the bloodstream occurs in patients with intestinal mucosa damage, immune deficiency and intestinal overgrowth [4]. Although *E. coli* is part of the commensal flora of the human intestine, there is a wide variety of pathogenic *E. coli* strains. These pathogens can cause diarrheal syndromes and are associated with characteristic virulence factors [5]. *E. coli* strains capable of causing bloodstream and urinary tract infections are known as extraintestinal pathogenic *E. coli* (ExPEC) [3].

Adequate antimicrobial susceptibility testing of clinical isolates is essential to guide antibiotic therapy. The Clinical and Laboratory Standards Institute (CLSI) [6][7] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [8] provide two of the most commonly used standards for susceptibility testing worldwide. While the use of EUCAST methodology is often preferred in Europe, CLSI methodology predominates in the United States [9]. The guidelines consist of breakpoints based on pharmacodynamic and pharmacokinetic properties of the antibiotics and data from clinical trials. Susceptibility to a certain antibiotic agent is determined *in vitro* by measuring the inhibition zone diameter of a disk diffusion test or by measuring the minimal inhibitory concentration (MIC) [8]. Although both refer to ISO 20776-1 standard broth microdilution method, there is an important methodological difference: while CLSI recommends a fixed 2:1 ratio of amoxicillin/clavulanic acid for MIC testing, EUCAST recommends a fixed concentration of 2 mg/L clavulanic acid. For susceptibility testing through disk diffusion, because of technical limitations, only a fixed ratio of both compounds is applied [10]. There are also important differences in the interpretation of amoxicillin/clavulanic acid MIC-results for *E. coli*: while CLSI provides three categories ("susceptible" (S), "intermediate" (I) and "resistant" (R)), EUCAST only applies R and S without an "I" category ("susceptible, higher exposure") [7] [11] [12]. This results in a low overall agreement for susceptibility data obtained through CLSI and EUCAST methodologies. As such, a higher percentage of isolates is reported as resistant, when the EUCAST methodology is applied [12]. In 2019, EUCAST introduced a new concept: the "Area of technical uncertainty" (ATU). The ATU entails one or more inhibition zone diameters of uncertain antibiotic susceptibility interpretation [13].

Increasing antibiotic resistance of *E. coli* and other bacterial species is a worldwide problem, causing many difficult-to-treat infections. This is, in part, a consequence of widespread overuse of antibiotics and the rapid spread of new resistance mechanisms [14]. As illustrated in figure 1, the susceptibility of *E. coli* blood isolates in our laboratory exceeded 90% between 1997-2005, with a strong decrease to 57% by 2018. Importantly, we substituted CLSI by EUCAST standards in 2014. Because both standards provide different breakpoints and manage the amoxicillin/clavulanic acid ratio in a distinct manner, the real evolution of *E. coli* amoxicillin/clavulanic acid susceptibility is difficult to evaluate. Therefore, we decided to retest isolates from 1985 onwards to evaluate the real evolution of susceptibility and unravel the influence of technical differences between both standards.

2. Material and Methods

The UZ Brussel is a tertiary care center with over 700 beds. Two hundred and thirty-seven non-duplicate clinical isolates were retrospectively selected for this study. These *E. coli* strains were isolated from clinical blood samples randomly selected between 1985 and 2018. All isolates were stored at -80°C. The included isolates are distributed over the years as follows: 1985 (n=11), 1990 (n=12), 1995 (n=13), 2000 (n=34), 2005 (n=33), 2010 (n=33), 2015 (n=50) and 2018 (n=51).

MIC-testing was performed by applying the ISO 20776-1 standard broth microdilution method, referred to by both EUCAST and CLSI M07-A11 methods [6][7]. Sensititre® Custom Plates were used, containing amoxicillin/clavulanic acid with a fixed concentration of 2 mg/L clavulanic acid, according to EUCAST guidelines. Additional plates contained a 2:1 ratio of amoxicillin/clavulanic acid, according to CLSI guidelines. MIC-tests were performed in cation-adjusted Mueller-Hilton inoculum broth (CAMH). Four or five colonies from overnight growth cultures on a blood agar plate were directly suspended in CAMH to match the turbidity of the 0.5 McFarland standard. Ten µL of this suspension was diluted 1:1000 with CAMH. Thereafter, 50 µL of the broth solution was added to each well of the 96 well plate with the help of the Sensititre® Automated Inoculation Delivery System (ThermoFisher, Waltham, U.S.), resulting in an inoculum of 5.10^5 CFU/mL. A plastic seal was placed over the panel to prevent dehydration. A blood agar was inoculated as well, in order to rule out contamination. The 96 well plate was then incubated for 18 ± 2 hours at 35 ± 1°C. Before reading the MIC, the positive control well was checked for growth. The 96 well plate was then placed in the Sensititre® Vizion (ThermoFisher, Waltham, U.S.) to read the results and to determine the MIC-values. *E. coli* ATCC25922 was tested with each series for quality control.

According to EUCAST, *Enterobacterales* with a MIC-value of ≤ 8/2 mg/L or > 8/2 mg/L are considered susceptible or resistant. According to CLSI, *Enterobacterales* with a MIC-value ≤ 8/4 mg/L are considered susceptible, while isolates with a MIC-value of 16/8 mg/L or ≥ 32/16 mg/L are considered intermediate or resistant.

Disk diffusion tests were performed on Mueller-Hilton agar with discs of amoxicillin/clavulanic acid (20/10 µg) (I2A, Montpellier, France). The Mueller-Hilton agar plates were incubated during 24 hours at 37°C. After incubation, the zone of inhibition was measured by a SIRscan® apparatus (I2A, Montpellier, France). The disk diffusion diameters were interpreted by using the EUCAST clinical breakpoints (S ≥ 19 mm, R < 19 mm).

From 30 *E. coli* strains, the genomic DNA was extracted using the Dneasy blood & tissue kit (Qiagen, Hilden, Germany) for Whole Genome Sequencing (WGS). DNA libraries were prepared via the KAPA Hyper Plus kit (Kapa Biosystems, Wilmington, MA, USA). All libraries were sequenced on a MiSeq instrument (Illumina, San Diego, CA, USA) using the v2 (2 × 250 bp) and v3 (2 × 300 bp) reagent kits. From 207 *E. coli* strains, genomic DNA was extracted using the Maxwell RSC Cell DNA purification kit (Promega Corporation, Madison, USA). Fragmentation of genomic DNA was carried out using the NEBNext® Ultra™ II FS module. Sequencing libraries, with an insert size of on average 550 bp, were prepared using the KAPA Hyper Plus kit (Kapa Biosystems, Wilmington, USA) and a Pippin Prep size selection. In order to avoid PCR bias, the PCR amplifications step was excluded and a 500 ng input of genomic DNA was used. After equimolar pooling, libraries were sequenced on a Novaseq 6000 instrument (Illumina, San Diego, CA, USA) using a SP-type flow cell with 500 cycli. The library was denatured and diluted according to the manufacturer's instructions. A 1% PhiX control library was included in each sequencing run. Sequence quality was assessed with FastQC (version 0.11.4) software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). De novo assembly of sequences obtained by both methods was performed using SPAdes genome assembler (http://bioinf.spbau.ru/spades). Identification of acquired beta-lactamase genes was performed using the ResFinder 4.1 database available from the Center for genomic Epidemiology (https://cge.cbs.dtu.dk/). The presence of resistance genes was determined with a minimum % identity threshold of 90% and a minimum length for coverage of 60%.

A chi-square test with alpha value 0.05 was used to verify differences in MIC-value distributions, obtained through EUCAST and CLSI testing procedures. The categorical similarity between MIC-testing and disk diffusion is described by agreement categories: agreement (identical categorical results with MIC-testing and disk diffusion), very major error (susceptible according to disk diffusion but resistant with MIC-testing) and major error (resistant according to disk diffusion and sensitive with

MIC-testing). A linear regression model with alpha value 0.05 was used to compare CLSI and EUCAST values over time. Finally, a chi-square test with alpha value 0.05 was used to compare genotypic data.

3. Results

According to EUCAST guidelines, 145/237 (61.2%) *E. coli* isolates were identified as susceptible (MIC ≤ 8/2 mg/L) and 92/237 (38.8%) as resistant (MIC > 8/2 mg/L) for amoxicillin/clavulanic acid. On the other hand, according to CLSI guidelines, 177/237 (75.7%) *E. coli* isolates were identified as susceptible (MIC ≤ 8/4 mg/L), 41/237 (17.3%) as intermediate (MIC = 16/8 mg/L) and 19/237 (8.0%) isolates as resistant for amoxicillin/clavulanic acid. A significant difference is observed in MIC-values, obtained through EUCAST and CLSI testing procedure (chi-square test, P=0.002) (figure 2).

When comparing MIC-values obtained through EUCAST and CLSI guidelines, a low overall agreement is observed. In only 130 of the isolates (55.0%), testing according to the EUCAST and CLSI criteria delivered identical MIC-values for amoxicillin/clavulanic acid. In 64 of the isolates (27.0%) the MIC-values diverged one dilution, in 38 (16.0%) two dilutions and in five (2.1%) three dilutions. Thirty-two isolates were defined as "susceptible" according to CLSI but were defined as "resistant" according to EUCAST. From these 107 discrepant results, testing according to EUCAST methodology revealed more resistant profiles in 93 *E. coli* strains (94.1%).

According to EUCAST guidelines, disk diffusion showed 144/237 (60.1%) amoxicillin/clavulanic acid susceptible and 93/237 (39.2%) resistant *E. coli* isolates.

Ten out of 237 isolates (4.2%) were defined as "susceptible" according to disk diffusion, but were defined as "resistant" according to their MIC-value. These isolates showed a MIC-value of 16 mg/L. Eight of these ten isolates were in the ATU that is located between inhibition zone diameters 19 and 20 mm. On the other hand, 13 out of 237 isolates (5.5%) were defined as "susceptible" by their MIC-value but were defined as "resistant" according to disk diffusion. These isolates showed a MIC-value of 8 mg/L. Overall, 43/237 (18.1%) of the isolates were found with an inhibition zone diameter corresponding with the ATU (figure 3). When comparing MIC-categorization (through EUCAST-recommended testing) to disk diffusion outcome, 90 % of the isolates had the same categorical outcome (R versus S) and the calculated kappa index was 0.802.

When analyzing the evolution of resistance over time with a linear regression model, EUCAST and CLSI methods showed a significant difference in resistance over the years (P=0.001) (figure 4). Due to the oscillating nature of the data over time and the limited sample size, with both techniques, no significant increase (P=0.069) in resistance over time could be demonstrated. However, there is clearly a trend towards significance. The linearity, normality and auto-correlation of the data as well as the variation of the model were checked with simple scatter plots and were considered acceptable.

Between 1985 and 2010, 86/136 (63.2%) of the isolates were susceptible according to EUCAST and 102/136 (75.0%) according to CLSI. Between 2015 and 2018, 59/101 (58.4%) of the isolates were susceptible according to EUCAST and 75/101 (74.2%) according to CLSI. There is no difference between susceptibility outcomes before and after our substitution of CLSI by EUCAST methodology in 2014 (chi-square test, P=0.897 for CLSI and P=0.382 for EUCAST).

WGS was successfully performed on 233 of the 237 isolates. When comparing MIC-values, obtained through EUCAST testing procedures, 83 out of 91 (91.2%) phenotypic resistant isolates and 44 out of 142 (31.0%) phenotypic susceptible isolates carried one beta-lactamase gene. When comparing MIC-values obtained through CLSI testing, 16 out of 19 (84.2%) phenotypical resistant isolates, 38 out of 41 (92.7%) phenotypical intermediate isolates and 74 out of 173 (42.8%) of phenotypic susceptible isolates carried one or two beta-lactamase genes. In total, eight resistant isolates (EUCAST testing) did not have an acquired beta-lactamase gene. In the 31 isolates determined as resistant by EUCAST guidelines and susceptible by CLSI guidelines, one or two beta-lactamase genes were found in 29 (93.5%) isolates

(Table 1).We identified four ESBL-genes (blaCTX-M-15, blaCTX-M-1, blaTEM-52C and blaTEM-54), two oxacillinases (blaOXA-1 and blaOXA-2), four inhibitor resistant beta-lactamases (blaTEM-206, blaTEM-126, blaTEM-34 and bla-TEM33) and six penicillinases (blaTEM-1A, blaTEM-1B, blaTEM-1C, blaTEM-1D, blaSHV-1 and blaCARB-2). Eleven isolates carried two beta-lactamase genes and 114 isolates carried only one gene (Table 2).

4. Discussion

In the current research paper we describe the evaluation of *E. coli* susceptibility over a period of almost four decades and investigate the role EUCAST and CLSI procedures. Amoxicillin/clavulanic acid MIC-values, obtained through EUCAST and CLSI testing, showed a low (55%) overall agreement. Application of CLSI guidelines results in a higher number of susceptible-classified *E. coli* strains, in comparison to EUCAST guided testing. This discrepancy is probably due to the high concentrations of clavulanic acid at a fixed ratio in the CLSI methodology. Delgado-Valverde *et al*., who also observed important discrepancies between EUCAST and CLSI amoxicillin/clavulanic acid MIC-values for *E. coli*, suggest that MIC-values obtained through EUCAST testing are more predictive of therapeutic failure [12]. Probably due to the oscillating nature of the data and the limited sample size, no significant increase in resistance over the years was observed, with CLSI nor with EUCAST guided testing. Although we observed no significant difference in resistance with our change from CLSI to EUCAST methodology, the higher inherent resistance profiles obtained through EUCAST testing will probably account for more isolates reported as resistant.

In our comparison of broth microdilution with disk diffusion amoxicillin/clavulanic acid susceptibility testing using EUCAST standard, most of the *E. coli* isolates (90.3%) showed the same categorical agreement (R or S). From the 23 discrepant results from disk diffusion testing, 10 are defined as very major error and 13 as major error. Our results highlight that, despite the additional cost and workload, it can still be useful to perform a MIC-test apart from a disk diffusion test. A MIC-value might proof invaluable when a result is situated in the ATU and when handling invasive isolates.

Several studies have already shown the usefulness of WGS in the study of antimicrobial phenotypic resistance [15]. In our study we highlight the discrepancies between EUCAST and CLSI phenotypic susceptibility testing. Interestingly, these discrepancies seem to correlate in part with detection of acquired beta-lactamase genes. While we detected one or more beta-lactamase genes in 91% of EUCAST-tested resistance isolates, we did only detect acquired beta-lactamase genes in 84.2% of CLSI-tested resistant isolates. Furthermore, EUCAST susceptible *E. coli* strains correlated better with the absence of beta-lactamase genes (69.0%), while CLSI susceptibility correlated in 57.2% of isolates without beta-lactamase genes present. To this regard, the intermediate susceptible category of CLSI guidelines provides some methodological difficulties for a straightforward comparison. Yet, most of EUCAST resistant / CLSI susceptible tested isolates (93.4%) harbored acquired beta-lactamase genes. These findings are consistent with the low agreement between both methodologies for MIC-value determination. Eight EUCAST and CLSI phenotypic resistant isolates showed no genes for an acquired beta-lactamase. This resistance profile could be explained due the presence of a point mutation in AmpC promotors, induction of chromosomal AmpC through antibiotic use or other non-acquired resistance mechanisms [16].

5. Conclusion

This study highlights the low agreement between EUCAST and CLSI methodologies when performing MIC-testing of amoxicillin/clavulanic acid. There is a higher degree of resistant-categorized *E. coli* strains, when EUCAST guidelines are applied. The low agreement between EUCAST and CLSI was confirmed by WGS, since most of EUCAST resistant / CLSI sensitive isolates harbored beta-lactamase

genes. This study included only *E. coli* isolates and should be extended to other *Enterobacterales* and other microorganisms.

6. Declarations

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Conflicts of interest/Competing interests: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Availability of data and material: Available

Code availability: Not applicable

Ethics approval: Not applicable

Consent to participate and for publication: Not applicable

Authors' contributions: VR, DT and PD contributed to the writing of the article. BN and CF performed the practical work. PD and DT helped with the design of the study. BK helped with the statistical analysis.

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