**Effect of initial bacterial inoculum density on zones of inhibition in disk diffusion antibiotic susceptibility testing**

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*Abstract* - **The primary objective of this study was to show how the density of the initial bacterial inoculum effects the zone inhibition in Disk diffusion Bauer Kirby (BK) antibiotic susceptibility testing. In this study, three strains of *Staphylococcus aureus* were tested: a methicillin sensitive clinical strain, a methicillin resistant clinical strain and ATCC 25923. A series of decreasing initial inoculum densities of the three tested strains were prepared and poured onto Mueller Hinton agar plates. After overnight incubation the zones of inhibition around tested antibiotics from different inoculum densities were measured in mm. The results showed that inoculum density does have an effect on the zones of inhibition in BK antibiotic susceptibility testing of *S. aureus* where in the case of gentamycin sensitivity category change occurred. Correlation analysis showed that there is significant negative correlation between tested inoculum densities and zones of inhibition clinical methicillin sensitive strain of *S. aureus* after using oxacillin and gentamycin (Pearson coefficient were -0.917 and -0.892, respectively), and between tested inoculum densities and zones of inhibition clinical methicillin resistant strain of *S. aureus* after using ampicillin (Pearson coefficient was -0.960). Hence, initial bacterial inoculum density can be of high relevance in Bauer-Kirby disk diffusion testing and ought to be precisely determined in purpose of adequate therapy ordination.**

 *Keywords****: Staphylococcus aureus, susceptibility testing, McFarland standards, antibiotics, zones of inhibition.***

1. **Introduction**

Antimicrobial susceptibility testing is a standardized procedure that testes a bacterial isolates sensitivity towards antibiotics. The most commonly used antibiotic susceptibility testing method is the Bauer Kirby disk diffusion method [1]. The results of the antibiotic susceptibility testing using Bauer Kirby disk diffusion method are evaluated based upon referent clinical breakpoints like EUCAST (European Committee on Antimicrobial Susceptibility Testing) and CLSI (Clinical and Laboratory Standard Institute). The test result depends upon a number of factors as: medium composition, pH, length of incubation, method of endpoint reading, as well as inoculum preparation [2]. Based on the interpreted test results adequate antibiotic therapy is ordinated.

A prerequisite for the disc-diffusion antibiotic susceptibility test is the adjustment of the initial bacterial inoculum density to a standardized scale of 0.5 McFarland [3]. The McFarland standard was developed in 1907, as an attempt to approximate the number of bacteria in a solution, by comparing a prepared bacterial inoculum density to a series of barium sulfate solutions [2]. The original McFarland standard is a chemical solution made by mixing barium chloride (Ba$Cl\_{2}$) and sulfuric acid ($H\_{2}SO\_{4}$) and results in formation of barium sulfate (Ba$SO\_{4}$) precipitate, which is the cause of turbidity. Table 1 shows a comparison between the McFarland standards, bacterial density and absorbance at wavelength of 600 nm [4]. Today, the 0.5 McFarland standard is used to approximate the bacterial number in saline solution for Bauer Kirby disk diffusion testing [5].

Table 1. McFarland Standard [6]

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **McFarland Standard** | **0.5** | **1** | **2** | **3** | **4** |
| **1.0% Barium chloride (ml)** | 0.05 | 0.1 | 0.2 | 0.3 | 0.4 |
| **1.0% Sulfuric acid (ml)** | 9.95 | 9.9 | 9.8 | 9.7 | 9.6 |
| **Approximate Bacterial****Suspension (ml)** | 1.5 x $10^{8}$ | 3.0 x $10^{8}$ | 6.0 x $10^{8}$ | 9.0 x $10^{8}$ | 1.2 x $10^{8}$ |
| **Absorbance** | 0.08 – 0.1 | 0.257 | 0.451 | 0.582 | 0.669 |

The Bauer Kirby disk diffusion method is also used in the oxacillin and cefoxitin screening tests for *Staphylococci*, as a marker for the detection of the mecA gene – which is the carrier of resistance to methicillin and many other antibiotics and as such is the cause of many difficult to treat infections [7]. Previous studies recommended the use of oxacillin [8], while recent studies recommend the use of cefoxitin for screening test [9]. According to the Clinical and Laboratory Standards Institute (CLSI) guidelines (2006) the use of cefoxitin screening test is considered more reliable for the detection of mecA gene [7].

Considering all this, the aims of our study we to evaluate the effect that different initial bacterial inoculum densities of tested *S. aureus* strains have on the results of BK disk diffusion method and if the initial bacterial inoculums cause a change in the bacterial resistance/sensitivity category, according to currently used guidelines, especially in the case of cefoxitin*.*

1. **Materials and methods**

The tested organisms included a total of three *S. aureus* strains: *S. aureus* ATCC 25923(Liofilchem, ViaScozia, Zona Industriale, Italy), a clinical strain of *S. aurues* that is methicillin sensitive and a clinical strain of the *S. aureus* that is methicillin resistant. The strains were kept in Lauria Bertani (LB) Broth supplemented with 50% glycerol at -80ºC. These bacterial strains were recovered from glycerol stocks by plating on Blood Agar Base. Species identification was carried out using standardized biochemical tests.

* 1. **Threat definition**

Initial bacterial inoculum densities were determined using a spectrophotometer at wavelength of 600nm. From the initial inoculums a series of 5 dilutions was made by transferring 200 µl from the first to the next tube(Initial inoculum, R1, R2, R3, R4). The OD of each dilution was measured on a spectrophotometer at wavelength of 600nm.

1. **Bauer Kirby Disk Diffusion Test**

The prepared initial inoculums and their serial dilutions were poured over Mueller Hinton agar plates, the antibiotic disks were added, and plates were incubated overnight at 37 ºC. The zones of inhibition which appeared around the disks were measured in millimeters (mm) and recorded.

1. **Antibiotic disks**

The tested antibiotic disks included: Ampicillin (Amp) (10 µg), Cefoxitin (Fox) (30 µg), Oxacillin (Ox) (1 µg) and Gentamycin (Gar) (10 µg) from the manufacturer Liofilchem.

1. **EUCAST Clinical Breakpoint Guideline**

EUCAST clinical breakpoints v.8.1. (2018) was used in the determination of sensitivity/resistance of the tested *S. aureus* strains (Table 2) for gentamycin, oxacillin and ampicillin, while a study from Swenson et al. 2005, and Anand et al. 2009, [7, 9] was used as a referee for the determination zones of inhibition for cefoxitin.

Table 2. EUCAST Clinical Breakpoints (Staphylococci) [10]

|  |  |  |
| --- | --- | --- |
| **EUCAST guidelines** | **Zone mm****S≥** | **Zone mm****R<** |
| **Oxacillin** | 20 | 20 |
| **Cefoxitin** | 20 | 19≤ |
| **Gentamycin** | 18 | 18 |
| **Ampicillin** | (Refer to cefoxitin screening test) | (Refer to cefoxitin screening test) |

1. **Detection of mecA gene**

Detection of mecA gene was determined according to Swenson et al. 2005, and Anand et al. 2009, [7, 9] was used as a referee for the determination zones of inhibition for cefoxitin using 30µg cefoxitin disks.

1. **Statistical Analysis**

Descriptive statistics and correlation analysis for data evaluation were performed using IBM Corp. Released 2017, IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.

1. **Results**

Measured zones of inhibition after overnight incubation and initial inoculum density of the 5 serial dilutions for all tested antibiotics for the clinical strain of *S. aureus* that is methicillin sensitive are shown in Table 3.

Table 3. Zones of inhibition of the clinical methicillin sensitive strain of S. aureus expressed in mm for all tested initial inoculum densities (S=Sensitive, R=Resistant, Abs= Absorbance)

|  |  |
| --- | --- |
|  | **Zones of inhibition expressed in mm** |
| **Antibiotic** | **Oxacillin** | **Cefoxitin** | **Gentamycin** | **Ampicillin** |
| Initial Inoculum(0.698 Abs) | 20 (S) | 28 (S) | 17 (R) | 25 (S) |
| R1 (0.266 Abs) | 22 (S) | 28 (S) | 23 (S) | 27 (S) |
| R2 (0.124 Abs) | 25 (S) | 31 (S) | 23 (S) | 32 (S) |
| R3 (0.026 Abs) | 24 (S) | 30 (S) | 23 (S) | 38 (S) |
| R4 (0.019 Abs) | 24 (S) | 31 (S) | 22 (S) | 38 (S) |
| **Mean ± Standard****deviation****(STDEV)**  | **23 ± 2.0** | **29.60 ±1.517** | **21.60 ±2.608** | **32 ± 6.042** |

According to the results obtained by BK disk diffusion testing using different densities of a clinical strain of *S. aureus* that is methicillin sensitive (according to cefoxitin screening test) no change in sensitivity category occurred when testing oxacillin, cefoxitin and ampicillin. A change in sensitivity category was recorded for gentamycin where at the initial inoculum density of 0.698 the strain was resistant towards the tested antibiotic and at lower initial inoculum concentrations it became sensitive. For oxacillin the zone of inhibition ranged from 20 to 25 mm, while for cefoxitin the zone of inhibition ranged from 17 to 23 mm for different inoculum densities. The highest difference in the measured zone of inhibition was recorded for ampicillin where it ranges from 25 to 38 mm for different inoculum densities.

Results obtained by antibiotic susceptibility testing of the methicillin sensitive clinical strain of *S. aureus* for all tested inoculum densities is shown in Table 4.

Table 4. Correlation between tested inoculum densities and zones of inhibition clinical methicillin sensitive strain of *S. aureus*

|  |  |  |  |
| --- | --- | --- | --- |
|  | **P value** | **Pearson Correlation** | **Zones of inhibition** |
| **Inoculum density** | 0.028 | -0.917 | **Oxacillin** |
| **Inoculum density** | 0.120 | -0.779 | **Cefoxitin** |
| **Inoculum density** | 0.042 | -0.892 | **Gentamycin** |
| **Inoculum density** | 0.053 | -0.873 | **Ampicillin** |

Significant negative correlation (p<0.05) is shown between tested inoculum densities and zones of inhibition clinical methicillin sensitive strain of *S. aureus* after using oxacillin and gentamycin, so higher values of density is going to result with lower zones of inhibition.

Results obtained by antibiotic susceptibility testing of the referent strain of *S. aureus* ATCC 25923 for all tested inoculum densities is shown in Table 5.

Table 5. Zones of inhibition of ATCC 25923 S. *aureus* expressed in mm (S=Sensitive, R=Resistant, Abs= Absorbance)

|  |  |
| --- | --- |
|  | **Zones of inhibition expressed in mm** |
| **Antibiotic** | **Oxacillin** | **Cefoxitin** | **Gentamycin** | **Ampicillin** |
| Initial Inoculum(0.674 Abs) | 27 (S) | 33 (S) | 22 (S) | 33 (S) |
| R1 (0.272 Abs) | 25 (S) | 33 (S) | 24 (S) | 33 (S) |
| R2 (0.113 Abs) | 27 (S) | 33 (S) | 24 (S) | 33 (S) |
| R3 (0.033 Abs) | 26 (S) | 32 (S) | 23 (S) | 34 (S) |
| R4 (0.016 Abs) | 26 (S) | 32 (S) | 24 (S) | 35 (S) |
| **Mean ± Standard deviation (STDEV)** | **26.20 ± 0.837** | **32.60 ± 0.548** | **23.40 ± 0.894** | **33.60 ± 0.894** |

No change in resistance category was recorded for the four tested antibiotics tested on *S. aureus* ATCC 25923. The zones of inhibition varied 1 to 2 mm for different inoculum densities when testing oxacillin and in 1mm for cefoxitin. Similar results were obtained for the other two tested antibiotics.

Table 6. Correlation between tested inoculum densities and zones of inhibition of ATCC 25923 S.*aureus*

|  |  |  |  |
| --- | --- | --- | --- |
|  | **P value** | **Pearson Correlation** | **Zones of inhibition** |
| **Inoculum density** | 0.597 | 0.322 | **Oxacillin** |
| **Inoculum density** | 0.225 | 0.660 | **Cefoxitin** |
| **Inoculum density** | 0.157 | -0.735 | **Gentamycin** |
| **Inoculum density** | 0.269 | -0.615 | **Ampicillin** |

As it is shown in Table 6, there is no significant correlation (p>0.05) between tested inoculum densities and zones of inhibition of ATCC 25923 *S. aureus*.

Results obtained by antibiotic susceptibility testing of a clinical strain of *S. aureus*, that is methicillin resistant (according to cefoxitin screening test), for all tested inoculum densities is shown in Table 7.

Table7.Zones of inhibition of clinical strain of S. *aureus* that is methicillin resistant, expressed in mm (S=Sensitive, R=Resistant, Abs= Absorbance).

|  |  |
| --- | --- |
|  | **Zones of inhibition in mm** |
| **Antibiotics** | **Oxacillin** | **Cefoxitin** | **Gentamycin** | **Ampicillin** |
| Initial Inoculum (0.708Abs) | 0 (R) | 14 (R) | 20 (R) | 9(R) |
| R1 (0.261 Abs) | 0 (R) | 19 (R) | 24 (R) | 10(R) |
| R2 (0.160 Abs) | 0 (R) | 17 (R) | 21 (R) | 11(R) |
| R3 (0.029 Abs) | 0 (R) | 18 (R) | 24 (R) | 11(R) |
| R4 (0.003 Abs) | 0 (R) | 17 (R) | 26 (R) | 11(R) |
| **Mean ± Standard deviation (STDEV)** | **/** | **17 ± 1.871** | **23 ± 2.449** | **10.4 ± 0.894** |

No changes in sensitivity category were registered for all the tested initial bacterial densities of the clinical MRSA strain for all tested antibiotics, however at the initial inoculum density of 0.261 yielded a zone of inhibition of 19mm, while a zone of inhibition of 14mm was recorded for the inoculum density of 0.708. Zones of inhibition for gentamycin ranged from 20 to 24 mm when testing gentamycin and from 9-11 mm when testing ampicillin on the same bacterial strain.

Table 8. Correlation between tested inoculum densities and zones of inhibition of a clinical strain of

*S. aureus* that is methicillin resistant

|  |  |  |  |
| --- | --- | --- | --- |
|  | **P value** | **Pearson Correlation** | **Zones of inhibition** |
| **Inoculum density** | / | / | **Oxacillin** |
| **Inoculum density** | 0.156 | -0.736 | **Cefoxitin** |
| **Inoculum density** | 0.131 | -0.767 | **Gentamycin** |
| **Inoculum density** | 0.010 | -0.960 | **Ampicillin** |

Significant negative correlation (p<0.05) is shown between tested inoculum densities and zones of inhibition clinical methicillin resistant strain of *S. aureus* after using ampicillin, so higher values of density is going to result with lower zones of inhibition.

1. **Discussion**

Antibiotic susceptibility testing, as one of the major tasks in clinical microbiology laboratories, is crucial in determining adequate therapy for patients suffering from bacterial infections. Due to cost savings the Bauer Kirby disk diffusion method is often applied. In this testing the results can be reported as quantitative data (zones of inhibition in mm), or a qualitative data (using categories susceptible, resistant and intermediate) [11].

A number of factors can affect the results of antibiotic susceptibility testing, using BK disk diffusion method, one of which is the density of the initial bacterial inoculum [2]. This is also very influential in other tests - like the cefoxitin screening test for the detection of mecA gene in *Staphylococci*, which is interpreted by the measured zones of inhibition around cefoxitin disks in mm [7]. The initial bacterial inoculum is usually determined using the McFarland standard [6, 15]. A number of factors can affect the results of antibiotic susceptibility testing, using BK disk diffusion method, one of which is the density of the initial bacterial inoculum [12, 13,14].

Considering this the aim of our study was to determine the effect of initial bacterial inoculums on the zones of inhibition in *Staphylococcus aureus* using BK disk diffusion method.

The results of our study indicate that in MRSA strains no change in the antibiotic sensitivity category was registered for all four tested antibiotics. On the contrary in the clinical strain of *S. aureus* that was methicillin sensitive a change in category was registered in the case of gentamycin- where higher inoculum densities gave smaller zones of inhibition which corresponded to R (resistant) category, while smaller inoculum densities gave larger zones of inhibition which corresponded to S (sensitive) category.

Correlation analysis showed that there is significant negative correlation between tested inoculum densities and zones of inhibition clinical methicillin sensitive strain of *S. aureus* after using oxacillin and gentamycin (Pearson coefficient were -0.917 and -0.892, respectively), and between tested inoculum densities and zones of inhibition clinical methicillin resistant strain of *S. aureus* after using ampicillin (Pearson coefficient was -0.960).

In our study methicillin resistance was determined according to cefoxitin screening test which was confirmed as more reliable for the determination of mecA gene presence than oxacillin screening test, that was in use before [7, 8, 9, 16]. In all the tested strains both cefoxitin and oxacillin screening tests yielded identical results regarding mecA gene detection.

The drawback of our study was that the made initial bacterial dilutions did not cover all the McFarland standards ranging from 0.5 to 5. Hence in our further studies we aim to determine change in sensitivity category compared to all McFarland standards from 0.5 to 5 and increase the number of tested bacterial isolates.

In clinical and diagnostic microbiology laboratories, phenotypic testing of bacterial antimicrobial resistance is widely used. The advantages of this well studied and standardized method are: low cost, easy to use and interpretation criteria readily available for commonly encountered organisms. Accuracy of the obtained results however depends on a number of factors, one of which is the use of a standardized inoculum density of 0.5 McFarland. Our study showed that the zone of inhibition in mm is affected by the inoculum density. The use of high density inoculums may lead to false results of resistance category which in the long run may further contribute to the loss of antibiotics that we are faced with today. That is why a study which tests and emphasizes the drawbacks of the commonly used testing procedures is crucial in avoiding interpretation errors. Hence, initial bacterial inoculum density can be of high relevance in Bauer-Kirby disk diffusion testing and ought to be precisely determined in purpose of adequate therapy ordination.

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