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Calibration of fusidic acid disk diffusion susceptibility testing of *Staphylococcus aureus*

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Single strain regression analysis, SRA, was used to calibrate disk diffusion fusidic acid susceptibility testing of *Staphylococcus aureus* in two laboratories using different standard methods but the same interpretative MIC limits. SRA equation constants were calculated using five different fusidic acid disk contents (1.5, 5, 15, 50, 150 μg). These disks were tested on five separate occasions against quality control strain *S. aureus* ATCC 29213. The National Committee for Clinical Laboratory Standards (NCCLS) method was employed in Tartu, Estonia (TE) and the Swedish Reference Group for Antibiotics (SRGA) method in Sweden at the Karolinska Hospital (KS). SRA constants obtained were used for calculating zone breakpoints corresponding to MIC breakpoints recommended by the SRGA (S ≤0.5 mg/L, R ≥1 mg/L). Zone diameter histograms from KS, performed with a 50 μg disk, and from TE, using a 10 μg disk, showed a clustering of wild type strains around 41 mm and 30 mm, respectively, reflecting differences in methodology. Zone breakpoints calculated from the equations were validated by comparison with the histograms. Breakpoints were also calculated for a suggested lower disk content in Sweden, 10 μg, and validated in tests of clinical isolates and by histogram analysis.

Key words: Fusidic acid; disc diffusion test; zone breakpoints; antibiotic susceptibility test; disc test calibration.

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Fusidic acid is a steroid-like antibiotic discovered in the early sixties with activity against Gram-positive organisms and which acts by impeding protein synthesis. Fusidic acid is primarily used for the treatment of staphylococcal infections (1–3), has been tested against infections with *Clostridium difficile* (4, 5), and is also recognized as a possible agent against mycobacterial infections (6). With increasing resistance among these and other Gram-positive species to other drugs, fusidic acid has emerged as a potentially important antibiotic. It is available as an oral drug and in the Nordic countries also for intravenous use. With this interest in fusidic acid as a therapeutic alternative there is a parallel demand for susceptibility test results. However, there are few recommendations given by reference authorities regarding MIC and zone breakpoints for fusidic acid and the recommended disk content varies. As part of an ongoing collaboration between Estonian laboratories and Sweden in susceptibility testing, the requirement for interpretative zone breakpoints for the available 10 μg disk was noted. In a recent publication such zone breakpoints have been given, corresponding to an MIC limit for susceptibility of S ≤0.5 mg/L (7). We used

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the possibility of calibrating the disk test with single-strain regression analysis, SRA (8, 9), and could confirm the results of Skov et al. (7). The present studies show that SRA calibration might serve as a convenient and useful tool for establishing interpretative zone breakpoints for susceptibility testing.

MATERIALS AND METHODS

Bacterial strains and culture media

Staphylococcus aureus ATCC 29213 was used as the reference and control strain in the two participating clinical laboratories, at the Institute of Microbiology, University of Tartu, Tartu, Estonia (TE), and at Karolinska Hospital, Stockholm, Sweden (KS). The reference strain was used both for quality control purposes with zone measurements around the routine test disk and for single strain regression analysis, SRA. In addition, in Tartu 130 clinical isolates of *S. aureus* were included for zone measurements using a 10 μg disk. A similar compilation of zone diameter values around the 50 μg disk for 3145 clinical isolates of *S. aureus* and 2775 isolates of coagulase-negative staphylococci was made at KS for histogram presentation. Thirty staphylococcal strains from a reference collection at the Swedish Institute for Infectious Disease Control (SMI) were also studied in detail at KS using SRA. This collection consisted of 10 strains of *S. aureus*, 6 strains of methicillin-resistant *S. aureus*, 7 strains of *Staphylococcus epidermidis*, and 7 strains of methicillin-resistant *S. epidermidis.* These strains represented a spectrum of MIC values for fusidic acid of from 0.064 to 256 mg/L. All strains were grown on Columbia blood agar base (Acumedia Manufacturers, Baltimore, through Svenska LabFab, Ljusne, Sweden), supplemented with 5% horse blood.

Disk diffusion susceptibility testing

Antibiotic susceptibility was determined using the disk diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS) (10) at TE and according to the Swedish Reference Group for Antibiotics (SRGA, http://www.srga.org) at KS (11). Fusidic acid disks containing 10 μg (TE) and 50 μg (KS) were obtained from Oxoid Ltd. (Basingstoke, England). The strains were inoculated onto Mueller Hinton agar (TE) and Iso-Sensitest agar (KS), both obtained from Oxoid Ltd. The bacterial inocula were 10^8 cfu/mL and 10^6 cfu/mL, respectively, and were applied by means of cotton swabs. Fusidic acid disks were placed on the inoculated surface followed by preincubation at room temperature for less than 15 min (TE) or 30 min (KS) before overnight incubation at 36°C±1°C in air. Inhibition zone diameters were read using a pair of calipers to the nearest millimetre either at the inner zone (TE) or at 80% inhibition of bacterial growth (KS). Interpretative zone breakpoints were only available in Sweden, using the 50 μg disk (S ≥30 mm, R ≤26 mm; SRGA, http://www.srga.org). The NCCLS provides no official information about fusidic acid susceptibility testing.

MIC determinations

The minimum inhibitory concentrations (MIC) of fusidic acid for the selected strains were determined using a standardised agar dilution method (11), in which fusidic acid was incorporated into Iso-Sensitest agar in two-fold dilutions. An inoculum of approximately 10^4 cfu per spot was applied by a multipoint inoculator. After incubation overnight at 36°C±1°C the MIC was read as the concentration where growth was judged to be completely inhibited. MIC limits for interpretation of susceptibility were S ≤0.5 mg/L, R ≥1.0 mg/L for fusidic acid (SRGA, http://www.srga.org). MIC of fusidic acid against strain ATCC 29213 was also determined using the Etest (AB Biodisk, Solna, Sweden).

Single strain regression analysis, SRA

The method "single strain regression analysis" was introduced in 1982 with the SRA equation which was developed from the classical equations describing the formation of inhibition zone in diffusion tests (12). In comparison with the regular regression line equation the SRA equation has retained the disk content as a variable which makes it possible to determine the constants of the equation using one strain and varying the antibiotic content in a series of disks. The equation can then be swapped to yield a regression line calculation and the zone diameters corresponding to various MIC values can be determined for various disk contents.

Antibiotic disks were produced in the KS laboratory with five different contents of fusidic acid: 1.5, 5.0, 15, 50, and 150 μg per disk. The empty paper disks used for the production of these series were obtained from Oxoid Ltd. The 30 selected strains were tested at KS and the reference strain *S. aureus* ATCC 29213 in both laboratories using these disks five times each on separate occasions. The test procedures followed recommendations for regular disk diffusion tests in the respective laboratories (8, 11, 12). Calculation of SRA equation constants A (slope constant) and B (the intercept) were performed using computer software (12).

RESULTS

Fusidic acid zone histograms of clinical isolates and reference strain

Inhibition zone diameters around fusidic acid 10 μg disks were measured for 130 clinical iso-

lates of *S. aureus* at TE as summarised in a histogram (Fig. 1A). The histogram showed a clustering of wild type strains around 22–35 mm (range 18–37 mm). The mean value of zone diameters for the reference strain ATCC 29213 (n=5) was 25.0 mm. When a similar histogram was made using results of routine clinical isolates at KS with a 50 µg disk, the susceptible population showed a maximum peak around 41 mm (Fig. 1B, 32–48 mm, with outliers). The mean value of strain ATCC 29213 (n=66) at KS was 39.1 mm, ±1.3. It was evident from the KS histogram that zone breakpoints recommened by SRGA (S ≥30 mm, R ≤26 mm) were appropriate for the KS laboratory from a statistical point of view.

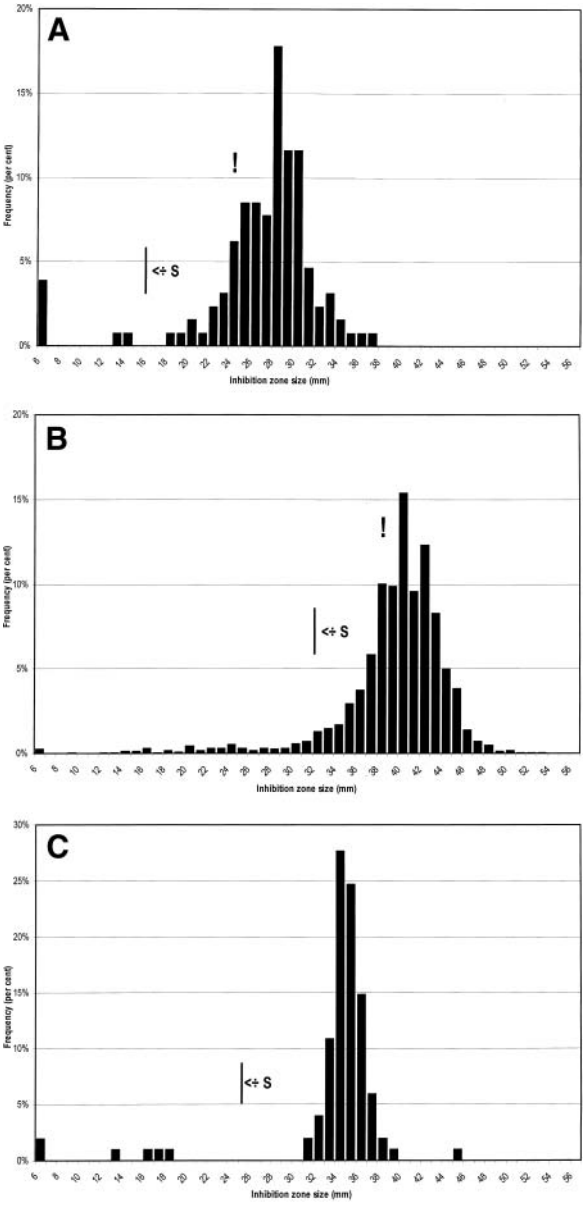

Fig. 1. Zone diameter histograms for clinical isolates of *S. aureus* and a 10 µg fusidic acid disk at Tartu laboratory (TE) (130 isolates, Fig. 1A), a 50 µg disk at KS (3145 isolates, Fig. 1B), and a 10 µg disk at KS (101 isolates, Fig. 1C). The mean values for reference strain *S. aureus* ATCC 29213 zone measurements were 25.0 mm (TE, Fig. 1A) and 39.1 mm ±1.3 (KS, Fig. 1B), respectively, in routine tests at the two laboratories (indicated by "!" in histograms 1A & 1B).

SRA studies of the reference strain S. aureus *ATCC 29213*

S. aureus ATCC 29213 was used in both laboratories as the reference strain for SRA calculations. In TE, the equation constants were: constant A=303, constant B=−113, and in the KS laboratory they were: A=521, B=22. The values of both constants were different in the two laboratories with constant A showing the greatest difference. This indicated that the performance of the two standard methods for disk diffusion would give different results. As a consequence, this precluded the use of SRGA recommendations in TE even if routine disks with the same content had been used in both laboratories. This was also clear from a comparison of fusidic acid zone diameter histograms at the two laboratories (Fig. 1A, 1B).

SRA studies of selected clinical isolates of staphylococci

Thirty strains of *S. aureus* and *S. epidermidis* covering a range of fusidic acid MIC values were also analysed using SRA at KS. Three strains, all with MIC 256 mg/L, could not be analysed because inhibition zones were only formed around the disk with the highest content. The constants obtained for the other 27 strains expressed as mean values were: constant A=497, constant B=137. When the isolates were grouped according to species and methicillin susceptibility, there were no significant differences between the constants in the four groups. The results were also analysed for differences between strains susceptible and strains resistant to fusidic acid. Seventeen staphylococcal strains with MIC values 0.032–0.5 mg/L were compared with 10 strains with MIC values 2–32 mg/L, and the median A constants were 552 (mean 558) compared with 397 (mean 394). This differ-

ence was significant with a p-value of 0.0156 (Chi-Square=5.85, df=1, Kruskal-Wallis median test). The regression line therefore seems to vary with the MIC values, a finding which earlier led to introduction of so-called standard curve regression analysis (13). A more practical way is to select a reference strain to be used for calculation of interpretative breakpoints separating the susceptible population of strains from resistant ones, with an MIC value in the susceptible range or around the MIC limits for optimal predictivity. This would ensure that the regression line constants used for calculating the interpretative breakpoints apply to this MIC interval. The ATCC 29213 reference strain suited this purpose.

Calculation of interpretative zone breakpoints using SRA

The SRA equation constants obtained using the *S. aureus* reference strain ATCC 29213 in the two laboratories were used for the calculation of interpretative zone breakpoints corresponding to recommended MIC limits (regression lines shown in Fig. 2). The exact procedure for the calculation followed earlier guidelines (14). First, the interpretative S breakpoint for the Estonian laboratory corresponding to the MIC limit for susceptibility (S ≤0.5 mg/L fusidic acid) was calculated to be S ≥16 mm. This zone breakpoint was included in the histogram of clinical isolates from Tartu and clearly separated the susceptible population from resistant strains (Fig. 1A). The zone breakpoint for the S category using a 50 μg fusidic acid disk at KS was then calculated to be S ≥32 mm. This zone breakpoint similarly separated the susceptible population of clinical isolates from resistant ones (Fig. 1B). A histogram comprised of 2778 clinical isolates of coagulase-negative staphylococci from KS during 1999 showed that this interpretative breakpoint also separated the susceptible population from an equally large population of resistant isolates (data not shown).

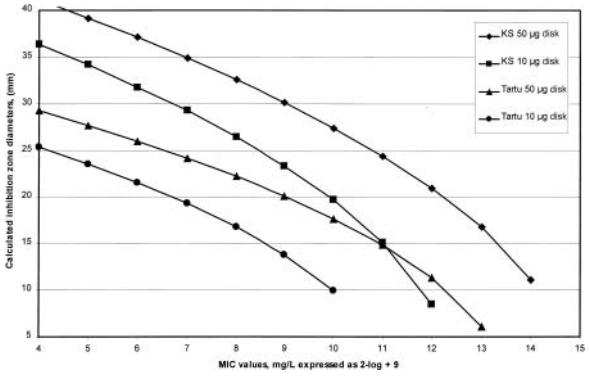

Fig. 2. Calculated fusidic acid regression lines based on results from single strain regression analysis, SRA, using *S. aureus* ATCC 29213 as the reference strain. The regression lines for the 50 μg and the 10 μg fusidic acid disk are shown as indicated for the Tartu as well as the KS laboratories.

Experiments were furthermore performed on the use of a 10 μg fusidic acid disk at KS. The calculated interpretative zone breakpoint for the susceptible category was S ≥25 mm. When this breakpoint was compared with the actual zones produced by clinical isolates around a 10 μg disk for the SRGA standardized procedure a clear separation of the susceptible population from resistant strains was achieved (Fig. 1C). The 50 μg disk recommended by the SRGA produced unnecessarily large inhibition zones (Fig. 1B). These studies suggested that a 10 μg disk, recommended by other reference authorities, could also be used according to the SRGA standardized procedure, following proper calibration.

SRA calculations using data from Skov et al. (7)

In the article by Skov et al. at Statens Serum Institut, SSI, Denmark (7), inhibition zone diameter results are given for the *S. aureus* ATCC 25923 strain for three different disk contents. Since the MIC of this strain is also given (a mean of 0.25 mg/L, using agar and microbroth dilution methods) a regression line can then be calculated for fusidic acid testing of *S. aureus* in their laboratory. The results are shown in Fig. 3 with regression lines given for the three disk contents, 5 μg, 10 μg, and 50 μg fusidic acid. Since the range of disk contents used by Skov et al. is only one 10-log, the curves have not been extrapolated as far as in Fig. 2, but the curve will include MIC limits recommended for the susceptibility categories. Using the calculation criteria for S and R zone breakpoints with SRA mentioned above (14) and the MIC limits S ≤0.5 mg/L and R ≥2 mg/L, the inhibition zone breakpoints were S ≥21 mm and R ≤19 mm with a 10 μg disk and S ≥20 mm and R ≤17 mm with a 5 μg disk. These values are

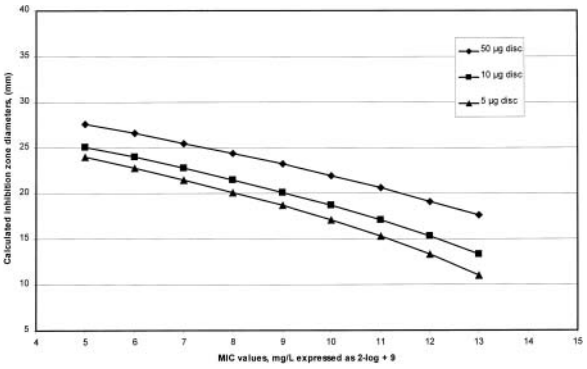

Fig. 3. SRA-calculated fusidic acid regression lines based on results published by Skov et al. (7), using *S. aureus* ATCC 25923 as the reference strain. The regression lines are shown for both the 5 µg and the 10 µg fusidic acid disk, but the extrapolation of the lines from the MIC of the strain, 0.25 mg/L, is restricted due to the narrow range of disk contents used for the results included in the calculations (7).

exactly the same (except for one zone breakpoint of 19 mm instead of 18 mm) as given by Skov et al. using the much more cumbersome method of testing 308 isolates and the error rate-bounded method (15). This analysis confirms the potential use of SRA for setting interpretative zone breakpoints in individual laboratories as well, thereby offering a true calibration of the disk diffusion method.

DISCUSSION

Recently, an investigation of fusidic acid disk diffusion testing using NCCLS methodology has been published giving quality control strain zone limits as well as inhibition zone breakpoint recommendations for both a 5 µg and a 10 µg disk (7). The NCCLS itself provides no official information about fusidic acid susceptibility testing, probably because this antibiotic is not approved for clinical use in the USA (16). This creates problems for laboratories in those countries where the drug is used and where the laboratories follow the guidelines approved by the NCCLS. The new recommendations by Skov et al. are therefore very welcome and it should be possible for laboratories to adopt these adhering to the NCCLS standard.

The fact that Skov el al. also provided zone diameter values for the control strain and different disk contents permitted the calculation of fusidic acid SRA regression lines for *S. aureus* in their laboratory setting (Fig. 3) (7). When interpretive zone diameter breakpoints were calculated from such SRA regression lines using the breakpoint setting method of Forsberg et al. (14) for the MIC limits S $\leq$0.5 mg/L and R $\geq$2 mg/L, the inhibition zone breakpoints were S $\geq$21 mm and R $\leq$19 mm with a 10 µg disk and S $\geq$20 mm and R $\leq$17 mm with a 5 µg disk. This is a remarkably precise result except for a one mm deviation when taken the zone breakpoints of Skov et al. as the true values. It suggests that SRA calculations could provide a powerful tool for both quality control and calibration of disk diffusion testing in individual laboratories as well. The studies by Skov et al. included 308 strains of *S. aureus*, whereas the SRA calculations were performed using results from one single reference strain.

When similar SRA studies were performed in the TE and KS laboratories, the resulting zone breakpoints were different from those obtained using the Skov et al. data at SSI. For KS this was expected since another standard is used for disk testing, the SRGA methodology; (http://www.srga.org) (11). For the TE laboratory the difference was not marginal (16 mm versus 21 mm) and suggested some differences between the NCCLS standard as applied in the TE and SSI laboratories. The differing zone breakpoint for TE was confirmed when compared with the zone histogram of clinical isolates of *S. aureus* strains tested in Tartu where the susceptible population was clearly separated from resistant isolates (Fig. 1A). The zone breakpoints by Skov et al. were therefore not useful for the TE laboratory, indicating that recommended interpretive breakpoints should preferably be evaluated in individual laboratories using zone histogram analysis and SRA.

A disk potency of 50 µg fusidic acid is presently used in Sweden for susceptibility testing. As shown in Fig. 1B the inhibition zones are excessively large. It was therefore of interest to evaluate the internationally accepted disc content of 10 µg fusidic acid using the SRGA disk test standardisation. The SRA-generated regression lines indicated rather shallow curves so a lower disk potency would not lower the zone sizes too much. This was confirmed in tests using a 10 µg disk at KS and the SRA-calculated interpretative zone breakpoint accurately

separated the susceptible population of strains from the resistant ones (Fig. 1C). This disk potency is more in line with a rational definition of an optimal disk content: "The smallest amount of antibiotic which will still distinguish resistant strains of any bacterial species from the intermediate or susceptible category by allowing zones of inhibition to be produced by all strains belonging to the latter categories" (17).

Calibration of the disk diffusion method in clinical microbiology laboratories is still an uncommon practice, in contrast to in clinical chemistry where calibration is a regular procedure. The accuracy of zone breakpoints issued by reference authorities has often been optimised by using the error rate-bounded method (15). However, when used on collections of isolates belonging to several different bacterial species, the results might be misleading if the collection of strains in the clinical laboratory, where the zone breakpoints are applied, does not reflect the same species selection. The DIN group applies the error rate-bounded method more correctly to individual species only (18). The investigation from SSI also used the error rate-bounded method (which they wrongly call the "error bound method") (7). As opposed to European authorities and contrary to mathematical principles they use the zone diameter values as the independent variable. Others have pointed out this error (19), but the NCCLS persists in following the wrong course. In the present studies we have demonstrated that direct calibration of the disk diffusion method is indeed possible using SRA (8). This method could be adopted in reference laboratories as well as in individual clinical microbiology laboratories to further improve the accuracy of the most commonly used method for antibiotic susceptibility testing, the disk diffusion method.

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REFERENCES

1. Verbist L. The antimicrobial activity of fusidic acid. J Antimicrob Chemother 1990;25 Suppl B: 1–5.
2. Coutant C, Olden D, Bell J, Turnidge JD. Disk diffusion interpretive criteria for fusidic acid susceptibility testing of staphylococci by the National Committee for Clinical Laboratory Standards method. Diagn Microbiol Infect Dis 1996; 25:9–13.
3. Anderson JD. Fusidic acid: new opportunities with an old antibiotic. CMAJ 1980;122:765–9.
4. Cronberg S, Castor B, Thorén A. Fusidic acid for the treatment of antibiotic-associated colitis induced by Clostridium difficile. Infection 1984; 12:276–9.
5. Wenisch C, Parschalk B, Hasenhundl M, Hirschl AM, Graninger W. Comparison of vancomycin, teicoplanin, metronidazole, and fusidic acid for the treatment of Clostridium difficile-associated diarrhea. Clin Infect Dis 1996;22:813–8.
6. Hoffner SE, Olsson-Liljequist B, Rydgard KJ, Svenson SB, Källenius G. Susceptibility of mycobacteria to fusidic acid. Eur J Clin Microbiol Infect Dis 1990;9:294–7.
7. Skov R, Frimodt-Möller N, Espersen F. Correlation of MIC methods and tentative interpretive criteria for disk diffusion susceptibility testing using NCCLS methodology for fusidic acid. Diagn Microbiol Infect Dis 2001;40:111–6.
8. Kronvall G, Ringertz S. Antibiotic disk diffusion testing revisited. Single strain regression analysis. Review article. APMIS 1991;99:295–306.
9. Kronvall G, Rylander M, Walder M, Lind-Brandberg L, Larsson P, Törnqvist E, et al. Calibration of disk diffusion antibiotic susceptibility testing: species-related trovafloxacin interpretive zone breakpoints and selection of disk potency. Scand J Infect Dis 1999;31:573–8.
10. National Committee for Clinical Laboratory Standards. Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard – Seventh Edition, M2-A7. NCCLS 2000.
11. Olsson-Liljequist B, Larsson P, Walder M, Miörner H. Antimicrobial susceptibility testing in Sweden. III. Methodology for susceptibility testing. Scand J Infect Dis 1997;Suppl 105:13–23.
12. Kronvall G. Analysis of a single reference strain for determination of gentamicin regression line constants and inhibition zone diameter breakpoints in quality control of disk diffusion antibiotic susceptibility testing. J Clin Microbiol 1982;16:784–93.
13. Ringertz S, Kronvall G. On the theory of the disk diffusion test. Evidence for a non-linear relationship between critical concentration and MIC, and its practical implications for susceptibility testing of *Haemophilus influenzae.* APMIS 1988;96:484–90.
14. Forsberg P, Petersson AC, Kronvall G. Determination of species- and laboratory-related interpretive breakpoints for doxycycline susceptibility

testing using single-strain regression analysis. Acta path microbiol immunol scand Sect. B 1985;93:53–60.
15. Metzler CM, DeHaan RM. Susceptibility tests of anaerobic bacteria: statistical and clinical considerations. J Infect Dis 1974;130:588–94.
16. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard – Fifth Edition, M7-A5. NCCLS 2000.
17. Kronvall G. Improving the accuracy of disk diffusion antibiotic susceptibility testing using species-related interpretive zone breakpoints. Indian J Med Microbiol 1998;16:138–42.
18. Schmalreck AF, Kottmann I, Reiser A, Ruffer U, Vanca E. In vitro testing of trovafloxacin according to the DIN 58940 guidelines: evaluation of trovafloxacin discs and proposal of tentative DIN breakpoints. Abstract no. P403. Clin Microbiol Infect 1997;3 Suppl 2:91.
19. Krasemann C, Hildenbrand G. Interpretation of agar diffusion tests. J Antimicrob Chemother 1980;6:181–7.