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► **To cite this version:**

Göran Kronvall, Christian G. Giske, Gunnar Kahlmeter. Setting interpretive breakpoints for antimicrobial susceptibility testing using disk diffusion. *International Journal of Antimicrobial Agents*, 2011, 38 (4), pp.281. 10.1016/j.ijantimicag.2011.04.006 . hal-00725395

HAL Id: hal-00725395

<https://hal.science/hal-00725395>

Submitted on 26 Aug 2012

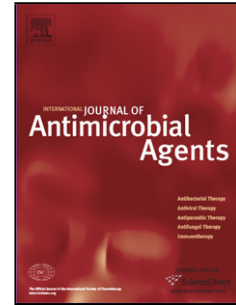
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Accepted Manuscript

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PII: S0924-8579(11)00196-8
DOI: doi:10.1016/j.ijantimicag.2011.04.006
Reference: ANTAGE 3608

To appear in: *International Journal of Antimicrobial Agents*

Received date: 2-4-2011
Accepted date: 5-4-2011

Please cite this article as: Kronvall G, Giske CG, Kahlmeter G, Setting interpretive breakpoints for antimicrobial susceptibility testing using disk diffusion, *International Journal of Antimicrobial Agents* (2008), doi:10.1016/j.ijantimicag.2011.04.006

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Setting interpretive breakpoints for antimicrobial susceptibility testing using disk diffusion

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ARTICLE INFO

Article history:

Received 2 April 2011

Accepted 5 April 2011

Keywords:

Antimicrobial susceptibility testing

Disk diffusion test

Interpretive breakpoints

Species-specific breakpoints

Epidemiological breakpoint

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ABSTRACT

Antimicrobial susceptibility testing plays a key role in clinical microbiology. The disk diffusion test dates back to the 1940s and became standardised from the 1950s, with the International Collaborative Study (ICS) and National Committee for Clinical Laboratory Standards (NCCLS) as the two major standards. Interlaboratory variation of disk test results was recognised early but has never been dealt with in a satisfactory manner. The error-rate bounded method was described in 1974 and its role is discussed. Species-specific susceptibility interpretation was coined in 1980 for *Proteus mirabilis* and chloramphenicol. In the late 1970s, more extensive use of species-specific breakpoints was introduced in Lund (Sweden). At the same time, P. Mouton constructed species-specific regression lines and pointed out the difficulties with narrow ranges of minimal inhibitory concentration (MIC) values. A more general use of species-specific regression lines was made possible with single-strain regression analysis, using one well-defined strain tested in disk diffusion with a range of disk contents. This method made it possible to calibrate the disk test in an individual laboratory. Other methods to achieve such calibration are also described. A recent method, 'MIC-coloured zone diameter histogram-technique', has proven useful for the validation of species-specific interpretive breakpoints. The microbiological breakpoint proposed by Williams in 1990 has experienced a renaissance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off value (ECOFF). MIC and zone diameter distributions with accompanying ECOFFs for species–antimicrobial combinations are published on the EUCAST website. A method for the reconstruction of wild-type zone diameter populations, namely normalised resistance interpretation, is described. This

method can produce resistance figures that are truly comparable between laboratories.

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1. Introduction

Soon after the discovery of antimicrobials, the problem of drug resistance among clinical pathogens became evident. Methods for the detection of antimicrobial resistance were soon set up in clinical microbiology laboratories [1–4]. The disk diffusion test, based on radial diffusion of antimicrobials from paper disks, proved well suited for testing pathogens isolated from clinical specimens in the routine microbiology laboratory. Two major standards emerged during subsequent years, one called the ICS method (International Collaborative Study) [5–7] and the other named the Kirby–Bauer disk diffusion method [8,9]. In the following decades, several complementary innovations have improved the accuracy of the disk diffusion test. At present, a major harmonisation of antimicrobial susceptibility testing is taking place in Europe [10]. A more detailed presentation of the historic developments in this area is provided in the Supplementary material. Herein we describe the developments regarding the disk diffusion method that have taken place during its 70-year history.

2. Classical methods to set interpretive breakpoints

A more extensive description of classical methods for setting breakpoints in disk diffusion testing is given in the Supplementary material. In principle, the major disk diffusion standards, Kirby–Bauer and ICS, relied on minimal inhibitory concentration (MIC) breakpoints for interpretation of susceptibility and translated these MIC limits into zone diameter breakpoints using the correlation between MIC values of bacterial isolates and corresponding inhibition zone diameters.

An example of a regression line is shown in Fig. 1, taken from a report on the ICS method by Ericsson and Sherris ($y = -2.78x + 48.49$) [6]. Their own interpretive breakpoints for the four susceptibility groups 1–4 in 1971 are shown in Fig. 1A, and the interpretive MIC breakpoints from the SIR system (susceptible, intermediate or resistant) introduced in Sweden in 1979 are shown in Fig. 1B [11]. A comparison of Fig. 1A and Fig. 1B illustrates the fact that interpretative rules and breakpoints may vary considerably.

An improvement introduced in 1974 was the error-rate bounded method of Metzler and DeHaan [12]. The method aimed at minimising the interpretive errors by comparing the true MIC values with the zone diameters and their interpretations. However, without a species-related aspect the error rates calculated do not reflect the real world. German scientists connected to the DIN reference authority (German Institute for Standardization) have used the error-rate bounded method species-wise, which is more correct [13]. For further aspects on this topic, see the Supplementary material.

3. Interlaboratory variation and its impact on susceptibility interpretation

Strict adherence to the standard as determined by the reference authority is the key to accurate interpretation and reproducible results of susceptibility testing. As long as there is no calibration method recommended for the individual laboratory, as in clinical chemistry, the control strain results should not only fall inside the range given by the reference authority but should also be randomly distributed inside the range.

The latter is often not clearly understood by laboratories. An analysis of the ability of control ranges to detect errors in laboratories using the National Committee for Clinical Laboratory Standards (NCCLS) standard was not very positive [14]. In an investigation of chloramphenicol susceptibility testing of *Haemophilus influenzae*, the interlaboratory variation was quite wide and the adherence to the Swedish Reference Group for Antibiotics (SRGA) standard was poor [15]. A follow-up study showed that a radical improvement of the standardisation had been achieved [16]. In a Canadian investigation of *H. influenzae* susceptibility testing in 66 laboratories, all of them claimed to follow the NCCLS standard but only 23 did so [17]. A lack of interlaboratory reproducibility was reported [17]. In a comparison of seven laboratories for marine microbiology, all claimed to adhere to an international standard in susceptibility testing. The intralaboratory precision showed a mean of 4.7% for the coefficients of variation (CV), whereas the interlaboratory precision was much lower with a mean of the CVs of 11.1% [18]. When 60 *Escherichia coli* reference strain results according to the NCCLS protocol for five agents in seven laboratories were analysed, 37% of the data sets contained >10% of their measurements outside the acceptable ranges [19]. Manninen et al. [20] in Finland analysed *E. coli* and *Staphylococcus aureus* resistance data before and after a change of the standard and concluded that the interlaboratory variation called for laboratory-specific interpretive breakpoints. The problem of interlaboratory variation has not been adequately dealt with by any reference authority and calls for further analysis and action [19,21,22]. Laboratories that measure and save their quantitative measurements, be they MIC values or inhibition zone diameters, are better equipped to compare data over time, to compare with other laboratories and to prove the validity of results in general and resistance surveillance trends in particular. Such

laboratories are also better equipped to re-interpret their data when breakpoints or methods change. These laboratories are also in the position to use epidemiological cut-off values as described below [23].

4. The general concept of species-specific interpretive breakpoints

Anecdotal comments in early papers indicated that occasional bacterial species did not follow the rules of the game. The term 'species-specific interpretive breakpoint' was coined by Furtado and Medeiros in an investigation of *Proteus mirabilis* and chloramphenicol susceptibility [24]. A high proportion of *P. mirabilis* isolates were assigned to the intermediate (I) category in disk diffusion tests, in contrast to their MIC values which suggested that they were susceptible. They concluded that "... species-specific breakpoints would more accurately predict the MIC equivalent of given zone diameters". John M. Matsen [25] had earlier studied carbenicillin susceptibility testing of *Pseudomonas aeruginosa*. Another disk was recommended as well as special breakpoints. He also used population distribution studies of zone diameter values for isolates in order to confirm breakpoint setting [25]. His findings were later confirmed by Fuchs et al. [26].

In 1976–1977, an extensive analysis of species-specific zone diameter distributions was carried out at the Clinical Microbiology Laboratory in Lund, Sweden, which identified a number of interpretive problems with the breakpoints recommended by the reference authority in Sweden, the SRGA [11,27]. Examples of the manual histograms produced in the laboratory are given in Fig. 2. As a result, from 1978 the laboratory in Lund used species-specific interpretive breakpoints [27]. A total of 11 species or species groups were defined with their own species-related interpretive

breakpoints. This pioneering move led to an improvement of the accuracy of antimicrobial susceptibility testing at this laboratory.

An illustration of the breakpoint problem with individual species is shown in Fig. 3 with results from Karolinska Hospital (Sweden) in 1983. The main *E. coli* population is apparently split by the recommended interpretive breakpoints of that time into a susceptible population from 26 mm and upwards, a resistant population from 20 mm and below, and with the intermediate population in between. It is obvious that this interpretation must be grossly wrong. However, *E. coli*, which for natural reasons made up such large proportions of Gram-negative isolates, were less prone to misinterpretation compared with other species. In the original description of the need for species-specific breakpoints, an example is given of an inhibition zone diameter histogram based on the repeat testing ($n = 45$) of a control strain of *P. mirabilis* against chloramphenicol. The interpretive breakpoint between susceptible (S) and resistant (R) divided the distribution of inhibition zones for the control strain [27]. One could just as well flip the coin to get the same results.

The idea of species-specific breakpoints was also understood in The Netherlands at about the same time [28,29]. Peter Mouton and his colleagues from the Dutch reference group, Commissie Richtlijnen Gevoeligheidsbepalingen (CRG), presented in 1981 a system for antibiotic sensitivity testing. In these documents they have included species-specific regression lines clearly showing that these regression lines were different for different pathogens [28,29]. The documents were written in Dutch and therefore did not reach the international community. In a 'Letter to the Editors', Mouton compared the Dutch standard with the SRGA standard and thereby brought

the Dutch recommendations to an international audience [30]. Mouton wrote that “.. breakpoints cutting through the middle of such populations have been avoided.” [30]. He concluded: “Thereby it becomes possible to report agar diffusion test results on the basis of species- or genus-specific regression functions. However, it is realized that without computer aid the routine use of such a system is laborious, impractical and prone to administrative errors.” [30]. They did not implement their knowledge because of this reasoning. On the contrary, we had no difficulties implementing the rather extensive breakpoint list in our laboratory in Lund. Actually, the laboratory technicians felt that these new species-related breakpoints were more consistent with their spontaneous categorisation according to their long experience of bacterial pathogens. Because of the Lund experience, a methodological subgroup of SRGA was formed in September 1987, SRGA-M, and this led to the issue of species-related interpretive breakpoint recommendations for Swedish laboratories [31,32]. The SRGA-M was lead by Gunnar Kahlmeter between 1987 and 2009. He was later appointed chairman of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) where he and his colleagues introduced species-related interpretations in Europe [23,33].

5. Species-specific regression analysis

When Peter Mouton and his colleagues calculated regression lines for individual species, they had problems with the mathematics when the range of MIC values noted for individual species was narrow [28,29]. Studies of the theoretical basis for the formation of a zone of inhibition in the disk test as it was described by Cooper in several publications [34] led to the insight that another way towards species-specific calculations of regression lines was possible [35]. This method was called single-

strain regression analysis (SRA). In principle, it uses one well-defined representative of the species studied with an accurate MIC value, preferably determined using intermediate dilutions as well [36], and in all known aspects showing the characteristics defining the species [35]. The chosen reference strain was tested in disk diffusion using several disk potencies and from these results the regular regression line could be deducted [35]. A computer program for the calculations can be downloaded free from <http://www.ki.se/labmed/clim/sra.htm>.

In Fig. 4, typical SRA regression lines are shown for *S. aureus*, *Enterococcus faecalis*, *E. coli* and *P. aeruginosa* tested against ciprofloxacin. The curved lines are due to the fact that SRA is performed using inhibition zone size squared for better linearity [34]. Considering the MIC limits given by reference authorities as the standard, the corresponding inhibition zone diameter breakpoints should be calculated separately for *P. aeruginosa* as its regression line is different from those of the other species. SRA makes it possible to do such separate calculations. It is very easily performed since no large collection of strains is required, just one well-characterised reference strain with a well-defined MIC value. SRA can be done in individual laboratories where interlaboratory variation otherwise would have resulted in erroneous interpretations. The SRA method has been applied to several combinations of antimicrobials and species [15,37–44]. A simple explanation of SRA with a review was published in 1991 [45].

A group in Finland has suggested an alternative method for the determination of species-specific regression lines, in this case for *Streptococcus pneumoniae* and tetracycline [46]. They used two reference strains with known MIC values, tested

them ten times and then drew a regression line from the means of the zone diameters and corresponding MIC values. This method might be of value in selected cases but lacks the more extended applicability of SRA calculations.

To the various applications of the SRA method should also be mentioned the possibility to determine an optimal disk content for disk diffusion tests. An optimal disk content could be defined as “The lowest disc content of antibiotic which will distinguish resistant strains of any bacterial species from strains of the intermediate or susceptible category.” [47,48]. SRA could therefore be used by reference authorities when determining the disk content of new antimicrobials for susceptibility testing. Other applications of SRA for testing disk contents were tried in Sweden and in Estonia on fusidic acid testing of *S. aureus* and the setting of breakpoints for use in Estonia [49]. An analysis on Iso-Sensitest Agar of various disk contents on gentamicin susceptibility testing of *E. faecalis* showed that a 30 µg disk could separate highly resistant strains from low-level resistant strains, thereby making an extra test using a 120 µg disk unnecessary [41]. In Sweden and some other countries, the standard gentamicin disk content is 30 µg, whereas the NCCLS/Clinical and Laboratory Standards Institute (CLSI) recommends a 10 µg gentamicin disk for species other than enterococci. Interpretive errors in disk diffusion susceptibility testing have been defined as error types I, II and III, and their remedies discussed, summing up some of the problems mentioned here [50].

6. Calibration of the antimicrobial disk diffusion test

The word 'calibration' is seldom heard among microbiologists but is always in the mind of the clinical chemist. To use in all other laboratories a breakpoint that is calculated in a reference laboratory is to a chemist something unheard of. One reason why we have never made laboratory-specific regression lines in clinical microbiology laboratories is the daunting workload caused by the need for testing many isolates of many species for many antibiotics covering a range of many MIC values. Such a procedure in the local laboratory would have provided laboratory-specific breakpoints, thus avoiding the impact of interlaboratory variation on susceptibility interpretations.

However, there are several alternative ways to determine more easily inhibition zone breakpoints in the individual laboratory. These methods will produce interpretive breakpoints that are both laboratory- and species-specific. While comparing results from two hospitals in different countries, O'Brien et al. [51] defined breakpoints in relation to the major susceptible populations, thereby calibrating the interpretations in a similar way in the two laboratories. This method is close to the first direct calibration method mentioned here, one we call reference strain calibration or peak correction [15]. Repeated testing of the reference strain used in quality control gives a mean zone value that is compared with the mean value of the same control strain in the reference laboratory [15]. The recommended zone breakpoint is then adjusted according to the deviation of the mean of the control strain compared with the reference laboratory mean. The peak-corrected breakpoints improved the accuracy from an overall incidence of false-resistant isolates of 4.4% to 2.3% in this early report [15].

The second method for calibration is a direct regression line calculation of individual species. It can be done in the classical way, as Mouton et al. have shown in The Netherlands [28–30]. The problems encountered were the poor coefficients of correlation obtained when the range of MIC values was narrow (typically 3–5 dilutions when no resistant organisms could be included), which is especially true for new drugs. A similar but quicker approach was described by Manninen et al. who used two reference strains for the species they studied with well-defined MIC values and then tested them ten times and drew regression line between these two points [20,46]. The interpretive zone breakpoint according to the MIC limit for susceptibility could then be calculated. Also here the breakpoints were both laboratory- and species-specific.

The third method for calibration is to use SRA [35,45,49]. As described above, this method requires only one single reference strain in order to define the regression line. With only 11 interpretive species/species groups this would only require 11 reference strains. These could be defined by reference authorities and sent to individual laboratories where interpretive problems have been identified, usually by using histogram analysis. Other examples of calibration using SRA, in addition to those given above, have been described [47–49].

In conjunction with the development of the EUCAST disk diffusion test method, several techniques for developing and provoking the validity of zone diameter breakpoints have been used. One of the most useful has been the ‘MIC-coloured zone diameter histogram-technique’, i.e. species-specific zone diameter distribution

histograms, as shown in Fig. 5a, where each isolate is represented in the zone diameter histogram in a colour representing its MIC value. By top loading the distribution with isolates containing resistance mechanisms, tentative zone diameter breakpoints can be validated. In Fig. 5b, the usefulness of the ceftazidime 30 µg disk for the detection of methicillin resistance in coagulase-negative staphylococci is shown, using a variant of the same technique. This technique has previously been used by the British Society for Antimicrobial Chemotherapy (BSAC) Working Party on Antimicrobial Susceptibility Testing (AST) and by the SRGA-M. EUCAST will make most of these analyses available on the EUCAST website.

7. Estimation of wild-type populations and cut-off values

Histogram analysis of inhibition zone diameter results was introduced early by O'Brien [51–53]. Independently, such analysis was performed in Lund as described above and provided the basis for the introduction of species-specific zone breakpoints in 1978 [27], and later in all Swedish laboratories by SRGA and SRGA-M [31]. The definition of breakpoints was made after visual inspection of the histograms and was easy when the distribution was unimodal or bimodal. An early advocate of this way of defining breakpoints was J.D. Williams, who suggested the use of 'microbiological breakpoints', defining the susceptible population and considering isolates outside this range as resistant [54]. He suggested to "...base the guidelines on the median or mode zone size of the susceptible population" and to set a "...microbiological guideline of 2 SD below the mode zone size (approximately 6 mm)..." [54].

The susceptible population is often referred to as the wild-type (WT) population, and the terms WT and non-WT (NWT) were proposed by EUCAST [23,33]. Recently, the terms epidemiological cut-off value (ECOFF) and clinical breakpoints have also been defined by EUCAST, with the ECOFF being identical to the microbiological breakpoint [23,33]. The presentation on the Internet by EUCAST (<http://www.eucast.org>) of WT MIC and inhibition zone diameter distributions of many combinations of antimicrobials and microorganisms from many investigators has been an important move for a better understanding of the value of histogram analysis. The distributions are used by breakpoint committees as one of several tools in the breakpoint-setting procedure. They are used as reference distributions by investigators, clinicians and those responsible for calibrating methodology. By sheer numbers (some of them consist of between 50 000 and 150 000 MIC values from over 100 investigators) they may in several instances be thought to define the 'true' MIC distribution. Furthermore, they are useful to determine whether or not a clinical isolate, irrespective of how it is categorised by a clinical breakpoint from EUCAST or CLSI, is devoid of phenotypically detectable resistance mechanisms.

When a single strain (a reference strain or a clinical isolate) is analysed repeatedly in disk diffusion tests, the aggregated inhibition zone values form a distribution most often covering 6–8 mm. When analysing a large number of isolates devoid of resistance mechanisms, the ensuing distribution is only marginally wider (under the same perfect conditions, 10–12 mm). This corresponds rather well to the distribution of susceptible isolates, the WT population. Such a distribution was analysed regarding conformity with a normal distribution and was found to fit well, except being slightly peaked and with a kurtosis towards higher zone values [41]. In fact, a WT

population might at a micro level even consist of isolates with slightly different means [55]. This most probably indicates some variation in growth or biochemical characteristics or some other still undefined factor [55]. Despite this microheterogeneity, the WT distribution does follow the normal distribution with the slight deviations mentioned. It is of interest that the microheterogeneity cannot be seen with two-fold dilution MIC tests. Only the higher precision of the disk test makes this heterogeneity visible [6,36,56].

The definition of a WT population by visual examination, although subjective, is easy and quite accurate. However, the emergence of resistance produces in some species and/or for some antimicrobials an unclear transition between the WT isolates devoid of detectable resistance and resistant isolates, which makes the decision on an epidemiological cut-off value rather subjective. There is a clear need for an objective method to describe and define the WT population of susceptible isolates in a histogram. The normalised resistance interpretation (NRI) method can fill this role [57–59]. It utilises the fact that log MIC and zone diameter distributions of the WT isolates are Gaussian in shape and that the lower half for MIC and the upper half for zones, i.e. the susceptible side of the peaks, should be unaffected by the occurrence of resistance and might therefore be used for reconstruction of the whole WT peak of the distribution. This is achieved by localising the peak using moving averages, then calculating the fraction of isolates from the highly susceptible side [58]. After probit transformation of these values, the resulting straight line can be solved by the least-squares method, defining a normal population corresponding to the WT peak [57,58]. In automatic calculations some parameters have to be set and in a given

comparative investigation these parameters should be kept the same throughout, making the results comparable [59].

Examples of NRI calculations are shown in Fig.6. In Fig. 6A, a zone diameter histogram of *E. coli* tested against a 30 µg gentamicin disk was subject to NRI and the resulting normal distribution shows a mean of 28.5 mm with a standard deviation (S.D.) of 1.75 mm. The 2.5 S.D. cut-off towards resistance gives $R < 24$ mm. In comparison with the interpretive breakpoint recommended in 2009, this gives a more conservative estimation of susceptibility and fits well with an visual determination of the WT peak. In Fig. 6B, a histogram of *E. coli* and ciprofloxacin is shown with the NRI-generated normalised distribution having a mean of 38.5 mm, S.D. of 3.15 mm and a 2.5 S.D. cut-off of $R < 30$ mm. This cut-off will include also zone diameter values of 24–29 mm among the non-susceptible ones, which is in accordance with previous studies showing the presence of quinolone resistance-determining region (QRDR) mutations among these strains [60]. The SRGA-recommended interpretive breakpoint of 2001 does not accommodate these circumstances that NRI so effectively takes care of.

NRI was critical in an investigation of antimicrobial resistance at Karolinska Hospital over a 30-year period [61]. During these years, the methodology for susceptibility testing had changed several times (change of medium, change of interpretive breakpoints, etc.), so a direct comparison of the interpretations was not possible. By analysing every annual histogram of *S. aureus* and *E. coli* zone diameters recorded, NRI analysis provided a comparable measure of resistance [61]. It was therefore possible to draw a temporal profile of resistance development. So far, few

laboratories have applied NRI. In aquaculture, special problems require active participation of individual laboratories. One problem is the low number of isolates tested. Smith et al. [62] have found that even small numbers of members of a given species might be enough to determine epidemiological cut-off values for susceptibility interpretation using NRI. NRI-generated epidemiological cut-off values in two different laboratories made comparison of resistance possible, whereas the regular breakpoints were inappropriate because of interlaboratory variation [63]. With these NRI-generated epidemiological cut-off values the results were comparable [63]. In a study of a reporter agent for quinolone antimicrobials, NRI calculations were also important [64].

NRI was originally developed for inhibition zone diameter histogram analysis but its use has later also been extended to MIC distributions. Tigecycline Etest results of isolates of 5 Gram-positive and 13 Gram-negative species from three university hospital laboratories were analysed using NRI, in total with 4771 isolates included [65]. With the intermediate MIC values of the Etest, enough data points were obtained for successful mathematical calculations [65]. Also, regular 2-log dilution MIC distributions have now been analysed using NRI, made possible by the introduction of helper variables [66]. *Staphylococcus aureus* and *E. coli* MIC distributions from the EUCAST website were included (27 of each species) and the NRI results were compared with the epidemiological cut-off values (ECOFFs) issued by EUCAST (http://www.eucast.org/mic_distributions/). The NRI-generated +2.0 S.D. values showed agreement with 26 of 27 within ± 1 dilution step and 17 exactly on the ECOFF values for *S. aureus*, and with 25 of 27 within ± 1 dilution step and 14 right on the ECOFF values for *E. coli*. The calculations were possible to perform on

distributions including numbers of isolates ranging from 40 to 124 472 [66]. Another method for the calculation of epidemiological cut-off values for MIC distributions has been presented by Turnidge et al. using an iterative statistical method [67].

8. General summary

During the 70-year history of antimicrobial susceptibility testing, methods have gone through several major phases of development. For decades, the principle for setting disk diffusion zone diameter breakpoints was first to define MIC breakpoints with due consideration of various aspects, such as pharmacokinetic, microbiological and clinical considerations. These MIC breakpoints were then translated into zone diameter breakpoints using a regression line between MIC values and inhibition zone diameters based on multiple species. The calculated zone breakpoints for an antimicrobial agent were intended for use irrespective of bacterial species. The two major standards were NCCLS/CLSI and ICS. The methods were improved on by the introduction of the use of the error rate-bounded method of Metzler and DeHaan. However, reports on shortcomings as well as pioneering work by O'Brien and by J.D. Williams and results from studies in Lund suggested that interpretations should be species-specific for improved accuracy. This more biological view with the definition of WT populations and epidemiological breakpoints was adopted by SRGA and the BSAC Working Party on AST and has then been further developed by EUCAST. Also, several methods developed for a more objective analysis of WT populations as well as species-specific regression analysis have been aiding in this transformation. With such objective methods also applied to MIC distributions and the advent of pharmacokinetic/pharmacodynamic–Monte Carlo simulations for defining MIC epidemiological breakpoints, it seems that this changing pattern has come full circle.

We now see the calculation of zone diameter breakpoints to match MIC epidemiological cut-off values (ECOFFs), using either species-specific regression analysis, repeat zone diameter testing of isolates representing MIC ECOFFs, or statistical methods to define both MIC and inhibition zone diameter ECOFFs.

Funding

None.

Competing interests

None declared.

Ethical approval

Not required.

Accepted Manuscript

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Fig. 1. Regression lines for tetracycline as given in [6] ($y = -2.78x + 48.49$). (A) The minimum inhibitory concentration (MIC) limits for the four susceptibility categories in 1971 are marked with green arrows: category 1, ≤ 1 mg/L; category 2, ≤ 8 mg/L; category 3, ≤ 32 mg/L; and category 4, > 32 mg/L [6]. (B) MIC limits for the SIR categories (susceptible, intermediate or resistant) in 1979 are shown in green with the corresponding zone diameter values marked with black arrows: category S (susceptible), ≤ 1 mg/L; and category R (resistant), ≥ 4 mg/L [11].

Fig. 2. Copy of hand-written histograms noted in the routine clinical microbiology service at Lund University Hospital (Sweden) in 1977 for *Escherichia coli* and *Proteus mirabilis*. The wild-type (WT) populations of these two species are marked by yellow for cefalothin, red for ampicillin and blue for nalidixic acid. The different locations of WT peaks for the two species makes general interpretive breakpoints impossible to use.

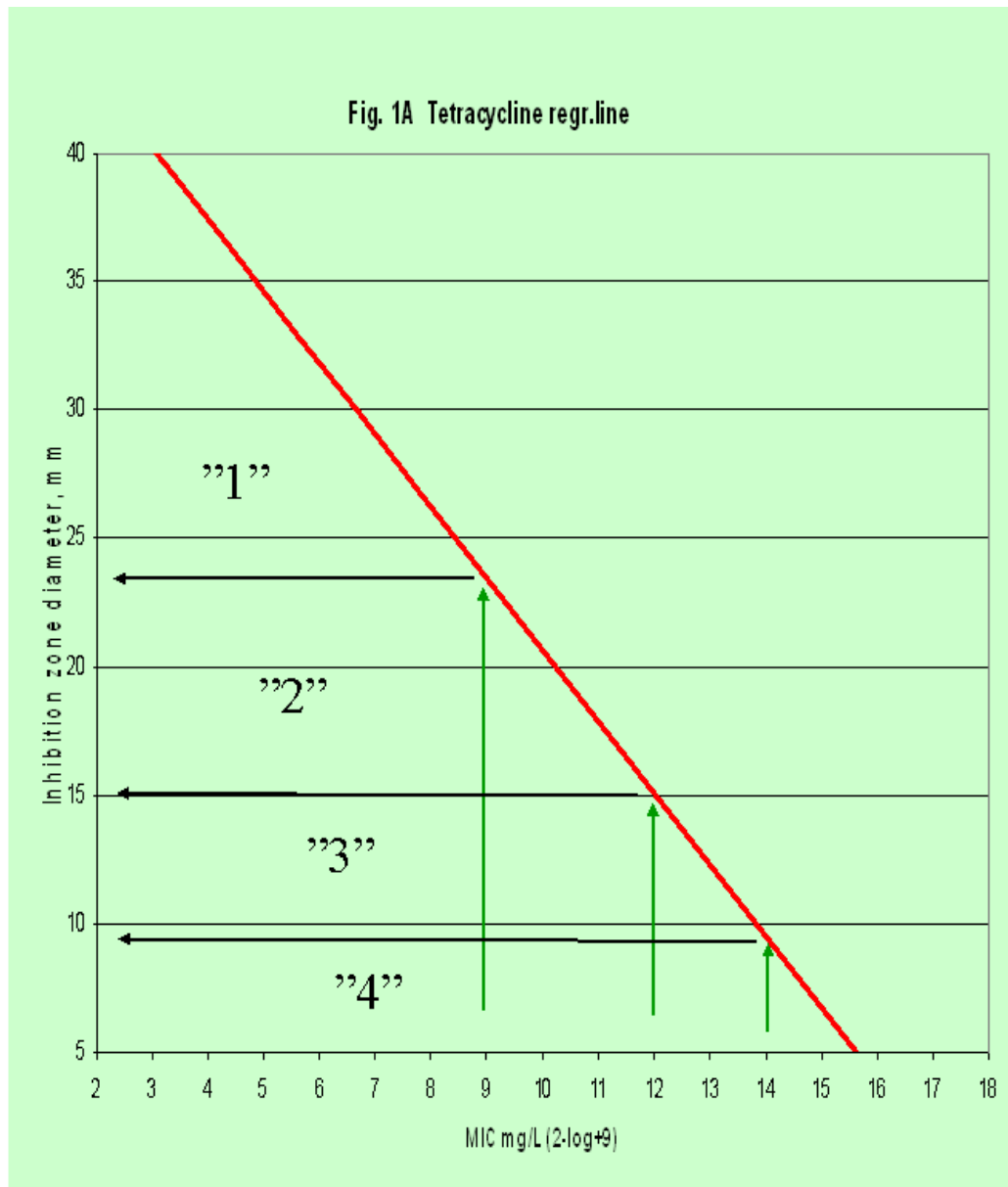
Fig. 3. Distribution of inhibition zone diameter values from disk diffusion tests of *Escherichia coli* with doxycycline disks at Karolinska Hospital (Sweden) in 1983. Susceptibility interpretations were performed using the zone breakpoints of that year, with susceptible (S) ≥ 26 mm (blue bars), intermediate (I) = 21–25 mm (green bars) and resistant (R) ≤ 20 mm (red bars). The main population of susceptible isolates is split by the breakpoints resulting in poor reproducibility of SIR categorisation (susceptible, intermediate or resistant) of organisms without resistance mechanisms to tetracyclines.

Fig. 4. Single-strain regression analysis (SRA) of *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* against ciprofloxacin. Calculations are performed using zone size squared for better linearity but then plotted on a linear zone scale. *Pseudomonas aeruginosa* is markedly different from the other three species. MIC, minimum inhibitory concentration.

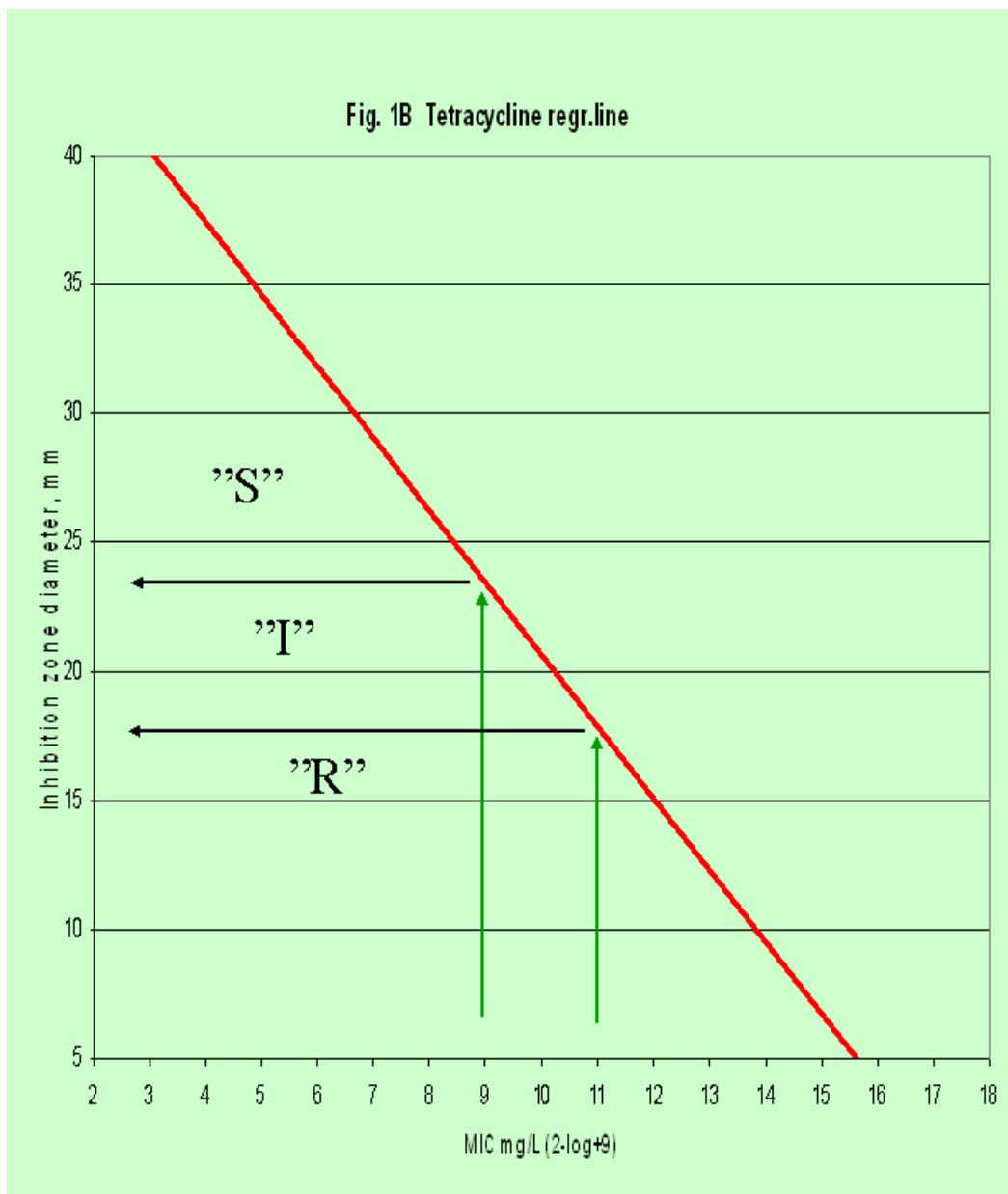
Fig. 5. 'MIC-coloured zone diameter histogram-technique': species-specific zone diameter distribution histograms, where each isolate is represented in the zone diameter histogram in a colour representing its minimum inhibitory concentration (MIC) value. (A) Zone diameter distribution of Enterobacteriaceae isolates tested against a 10 µg gentamicin disk is shown, with the MIC values of the isolates indicated by colours. (B) The usefulness of the 30 µg ceftiofur disk for the detection of methicillin resistance in coagulase-negative staphylococci is shown.

Fig. 6. Inhibition zone diameter histograms are shown for *Escherichia coli* and gentamicin in 2009 (A) and ciprofloxacin in 2001 (B). Blue bars indicate susceptible isolates, green bars intermediate isolates and red bars resistant isolates, as defined by Swedish Reference Group for Antibiotics (SRGA)-issued interpretive zone breakpoints at the time. Normalised resistance interpretation (NRI)-calculated wild-type (WT) populations are shown in red curves. The NRI-calculated non-WT (NWT) limits are resistant (R) <24 mm for gentamicin and R <30 mm for ciprofloxacin. These should correspond to epidemiological cut-off values (ECOFFs) determined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) of ≤2 mg/L for gentamicin ($n = 43\,924$ MIC values from 93 data sources) and ≤0.032 mg/L for ciprofloxacin ($n = 17\,877$ MIC values from 82 data sources).

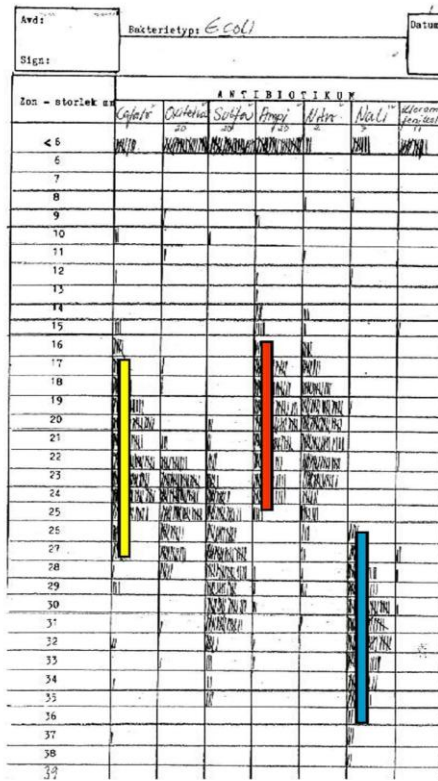
(A)



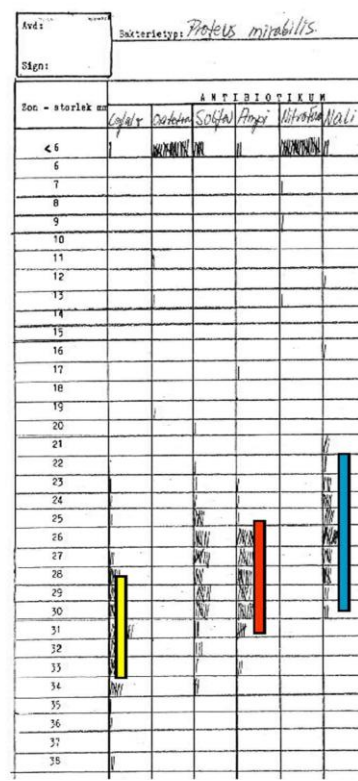
(B)



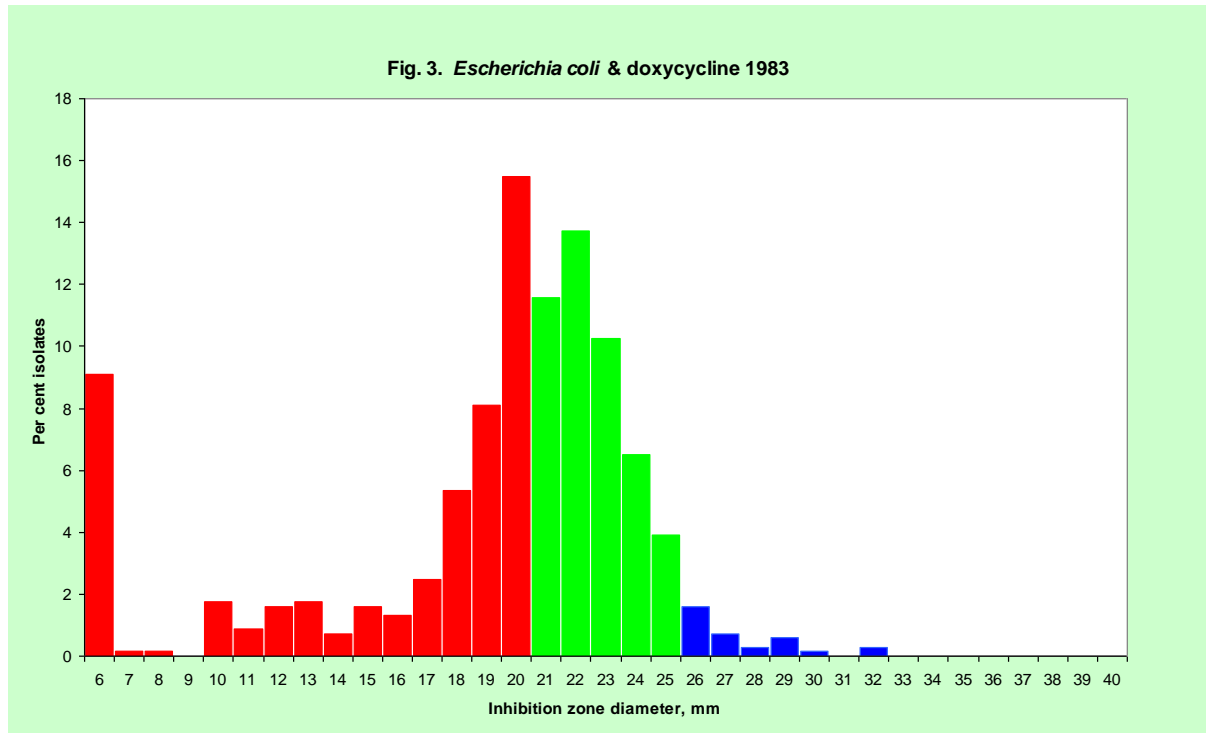
Escherichia coli



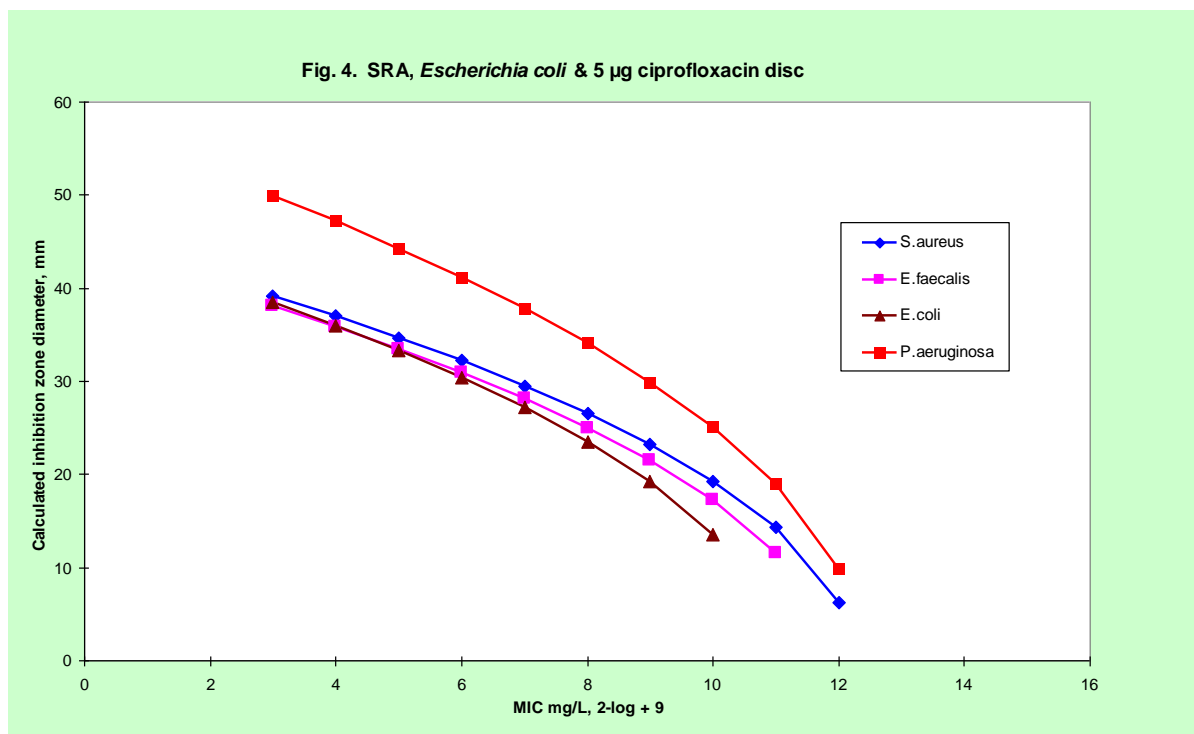
Proteus mirabilis



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