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# Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method

**Boyan Bonev*, James Hooper and Judicaël Parisot**

*School of Biomedical Sciences and Institute of Infection and Immunity, University of Nottingham, Nottingham NG7 2UH, UK*

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***Objectives*: The agar diffusion assay is one method for quantifying the ability of antibiotics to inhibit bacterial growth. Interpretation of results from this assay relies on model-dependent analysis, which is based on the assumption that antibiotics diffuse freely in the solid nutrient medium. In many cases, this assumption may be incorrect, which leads to significant deviations of the predicted behaviour from the experiment and to inaccurate assessment of bacterial susceptibility to antibiotics. We sought a theoretical description of the agar diffusion assay that takes into consideration loss of antibiotic during diffusion and provides higher accuracy of the MIC determined from the assay.**

***Methods*: We propose a new theoretical framework for analysis of agar diffusion assays. MIC was determined by this technique for a number of antibiotics and analysis was carried out using both the existing free diffusion and the new dissipative diffusion models.**

***Results*: A theory for analysis of antibiotic diffusion in solid media is described, in which we consider possible interactions of the test antibiotic with the solid medium or partial antibiotic inactivation during diffusion. This is particularly relevant to the analysis of diffusion of hydrophobic or amphipathic compounds. The model is based on a generalized diffusion equation, which includes the existing theory as a special case and contains an additional, dissipative term.**

***Conclusions*: Analysis of agar diffusion experiments using the new model allows significantly more accurate interpretation of experimental results and determination of MICs. The model has more general validity and is applicable to analysis of other dissipative processes, for example to antigen diffusion and to calculations of substrate load in affinity purification.**

Keywords: agar diffusion assay, antibiotic activity testing, antibiotic resistance

## Introduction

Accurate determination of bacterial susceptibility to antibiotics is essential to the successful management of bacterial infections and to the comparative analysis of antimicrobial agents. This can be done by a number of techniques, which include the disc diffusion method, the broth dilution assay and the Etests. The effectiveness of antibiotics can be assessed by their ability to suppress bacterial growth, described by the MIC, or by their ability to kill bacteria, characterized by the minimal lethal concentration (MLC). MIC is usually derived by means of tests in solid media, whereas both MIC and MLC can be determined in broth dilution assays. A number of reports have been dedicated to comparing the effectiveness of these methods.[1–3]

The agar diffusion technique[4] is commonly used for determination of MIC in solid media. It involves the application of antibiotic solutions of different concentrations to cups, wells or paper discs, placed on the surface of or punched into agar plates seeded with the test bacterial strain. Antibiotic diffusion from these sources into the agarose medium leads to inhibition of bacterial growth in the vicinity of the source and to the formation of clear 'zones' without bacterial lawn. The diameter of these zones increases with antibiotic concentration. The value of MIC is determined as the zero intercept of a linear regression of the squared size of these inhibition zones, $x$, plotted against the natural logarithm of the antibiotic concentration, $c$:

$$\ln(\mathrm{MIC}) = \ln(c) - \frac{x^2}{4Dt} \quad (1)$$

Here, $D$ is the diffusion coefficient, presumed to be independent of concentration,[5] and $t$ the time of antibiotic diffusion.

*Corresponding author. Tel: +44-115-823-0177; Fax: +44-115-823-0142; E-mail: boyan.bonev@nottingham.ac.uk



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This analysis is based on a solution of a differential equation describing free diffusion in one dimension:[6–8]

$$D\frac{\partial^2 c(x,t)}{\partial x^2} - \frac{\partial c(x,t)}{\partial t} = 0 \qquad (2)$$

where $c(x,t)$ describes the dependence of antibiotic concentration on distance from the source and on time. This approach has been applied successfully to studies of two-dimensional diffusion of dyes and antibiotics, most notably penicillins.[5]

Significant deviation from the described behaviour is observed during the analysis of diffusion in solid agar of a number of antibiotics, especially those of more hydrophobic or amphipathic nature. One notable example is subtilin, where the size of inhibition zones increases linearly, rather than quadratically with the logarithm of antibiotic concentration.[9] Such deviations from the predicted behaviour have been addressed in different ways. In some cases, as in the subtilin example,[9] this has been taken into consideration and diffusion data have been analysed using $x$ versus $\ln(c)$ plots to determine MIC, even in the absence of theoretical justification for the use of this approach. Alternatively, the non-linearity has been absorbed in the approximation and a straight line has been fitted to the squared zone size versus $\ln(c)$, which results in some cases in overestimated values of the MIC. The efforts to analyse accurately and reproducibly the data from agar diffusion assays have, also, led to the development of sophisticated statistical tools.[10] Using the agar diffusion method, calibration zones can be introduced,[11] which demarcate ranges of susceptibility, resistance or refer to more complex responses of bacteria to antibiotic agents.

We consider the principles of antibiotic diffusion in solid media and propose a new theoretical model for data analysis, in which we take into account possible loss of antibiotic during the diffusion process. In the limiting case of lossless diffusion, this approach is equivalent to the existing free diffusion model. It allows a more accurate quantitative assessment of bacterial susceptibility to a wide range of antimicrobial agents, the diffusion of which has shown deviation from the free diffusion behaviour. Among other factors, such dissipative diffusion may result from antibiotic interactions with the diffusion medium, antibiotic degradation, antibiotic removal by the bacterial film or other loss of substrate during diffusion. We propose a theoretical model based on diffusion in one dimension, in which we distinguish cases of free diffusion from cases of dissipative diffusion. We provide justification for the use of either linear or quadratic functional dependence of inhibition zone sizes on the natural logarithm of antibiotic concentration and the choice of model can be made using the value of the regression coefficient $R^2$, which is closer to 1. Our model is also applicable to the analysis of other processes of dissipative diffusion, for example, antibody diffusion or resin loading during affinity purification, where the substrate binds to the medium during the loading process.

We have used our approach to investigate the diffusion in solid agar medium of nisin and subtilin (class IA lanthionine antibiotics), of the macrolide erythromycin, of tetracycline, of vancomycin (a glycopeptide), of the aminoglycosides kanamycin and gentamicin and of ampicillin, a β-lactam antibiotic (Figure 1). We compare the dependence of inhibition zone sizes on antibiotic concentration and the calculated MIC using both models.

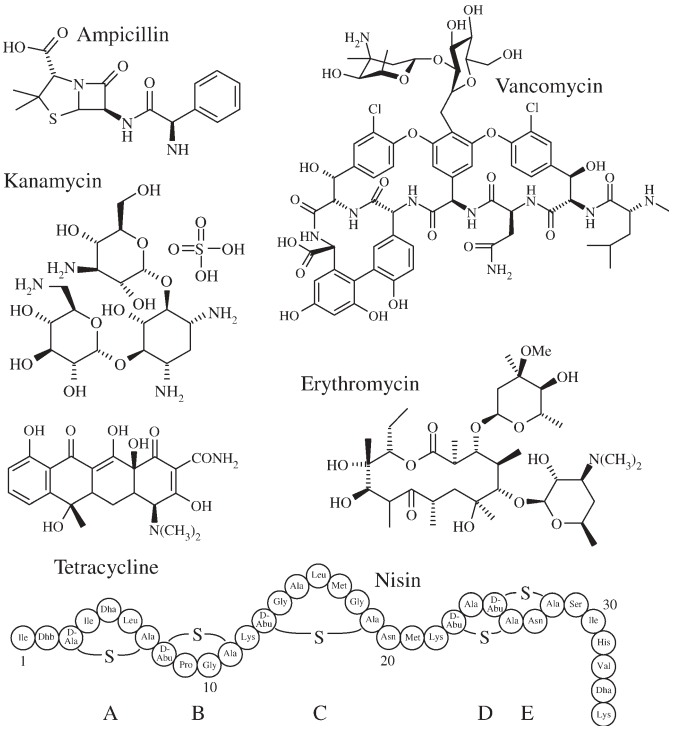


**Figure 1.** Chemical formulae of representative antibiotics from each group: ampicillin, a penicillin; vancomycin, a glycopeptide; tetracycline; kanamycin, an aminoglycoside; erythromycin, a macrolide; and nisin, a lanthionine antibiotic.

## Materials and methods

Chemicals were of analytical grade or better. Antibiotics were purchased at microbiological or analytical grade from Duchefa, Haarlem, The Netherlands (vancomycin, tetracycline, gentamicin and erythromycin), from Melford, Ipswich, UK (ampicillin) or Sigma-Aldrich, Steinheim, Germany (kanamycin) and were used without further purification. Bacterial strains were a kind gift from Nikki Horn and Mike Gasson, BBSRC, IFR, Norwich, UK. Nisin was produced and purified from streptomycin/rifampicin-resistant *Lactococcus lactis* F15876, as described previously.[12] Subtilin was produced from *Bacillus subtilis* ATCC 6633 and purified following a similar method.[13] MIC determinations were carried out by agar diffusion assay[6] against streptomycin- and rifampicin-resistant *L. lactis* MG1614,[14] which is susceptible to nisin. Bacteria for lawn seeding were grown in liquid GM17 medium. Inocula were spread on solid agar GM17 plates—37.5 g/L M17 (Difco), 15 g/L Microagar (Duchefa), 0.5% glucose, 200 μg/L streptomycin, 40 mg/L bromocresol purple. Standard 6 mm paper discs were placed on the surface of the agar and 25 μL of antibiotics at the desired concentration was added. Instead of discs, 3 mm holes were punched with a glass capillary and were filled with the same amount of nisin and subtilin solutions. Diffusion distances were determined as the half of the inhibition zone diameter less the disc or well diameter. Plates were analysed individually to determine MIC and the average values from three repeats were taken in determination of the final MIC. This was done to ensure that all inhibition zones within each experiment were obtained under the same experimental conditions. Initial data analysis was carried out using Microsoft Excel spreadsheet and a web tool at http://www.agardiffusion.com.



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## Results

### *Theory*

Assays of bacterial susceptibility to antibiotics by the agar diffusion method are analysed using linear fitting of the squared radius (diameter) of the inhibition zones to the natural logarithm of antibiotic concentration at the source [equation (1)]. This reflects a solution of the differential equation describing free diffusion in one dimension [equation (2)]. However, agar diffusion assays of nisin, tetracycline, gentamicin and kanamycin show dependence of zone size on ln($c$), which is better described as linear, rather than the predicted quadratic relationship. Indeed, previous experiments with subtilin diffusion in agar plates have been analysed using a linear fit.[9] The molecules of nisin and subtilin are amphipathic by nature, whereas tetracycline and gentamicin are relatively hydrophobic. This observation led us to consider the possibility that these antibiotics may diffuse through the agar medium more slowly than predicted by the free diffusion model. We propose an alternative model of diffusion, in which some of the antibiotic molecules may interact with the diffusion matrix or be lost through another dissipative mechanism. In order to take into account such loss of substrate, we propose the introduction of a dissipative term in the diffusion equation. The dissipative diffusion equation then becomes:

$$D\frac{\partial^2 c(x,t)}{\partial x^2} + V\frac{\partial c(x,t)}{\partial x} - \frac{\partial c(x,t)}{\partial t} = 0 \qquad (3)$$

where $V$ is a coefficient characterizing the dissipation rate.

One possible solution of equation (3) can be sought by the method of separating variables. If we assume that the concentration distribution, which is a function of distance and time, can be expressed as a product of two functions, each dependent on $x$ or $t$ only, we can re-write equation (3) in a separated variables form. This leads to two ordinary differential equations, one describing the time dependence of concentration and the other—its variation in space.

The general form of the space part of the solution shows diffusion as absorption-dominated and exponentially decaying, which can be expressed for the case of semi-infinite medium as:

$$\ln(MIC) = \ln(c) - (2D)^{-1}\left(V \pm \sqrt{V^2 - 4D}\right)x \qquad (4)$$

This result describes an exponential reduction in the amount of material, available for diffusion, which might be due to binding of antibiotic to the agarose matrix, degradation or another mechanism. In essence, when the dissipative term dominates, i.e. $V^2 >> 4D$, we observe an exponential decrease in concentration with distance. In the vicinity of $V^2 = 4D$, equation (4) gives rise to a solution that can be converted for vanishing $V$ to a solution obtained from the free diffusion equation by separation of variables.[15]

### *Experiment*

Assays of *L. lactis* MG1614 susceptibility to antibiotics were carried out using diffusion in solid agar medium of ampicillin, vancomycin, tetracycline, nisin, subtilin, gentamicin, kanamycin and erythromycin. The experimental results were analysed using the absorptive model, described here, as well as by the existing free diffusion model. The susceptibility values in each case were within the same order of magnitude but differed by more than 100%. Experimental MIC values, determined using absorptive (linear) and free (quadratic) diffusion are summarized in Table 1 together with the corresponding $R^2$ values from the regression analysis. Values of $R^2$ closer to 1 are obtained from a better fit.

The size of inhibition zones is presented as a linear or quadratic function of the natural logarithm of antibiotic concentration together with linear fits and regression residues for each antibiotic. Diffusion of ampicillin and vancomycin (Figure 2) was best described using the conventional, free diffusion model, which suggests little interaction between these antibiotics and the diffusion medium. The corresponding $R^2$ values were 1.000 and 1.000 for free diffusion and 0.977 and 0.986 for absorptive diffusion. There is approximately 2-fold difference in the corresponding MIC values (Table 1).

In contrast, the diffusion in agar of the amphipathic antibiotics nisin and subtilin and the relatively hydrophobic molecules of tetracycline are best analysed using the absorptive model (Figure 3), giving $R^2$ of 0.998 and 0.999 from the $x$/ln($c$) fit and

**Table 1.** Susceptibility of *L. lactis* MG1614 to antibiotics: MIC (mg/L) and $R^2$ values from linear regression analysis using quadratic or linear dependence of zone size (mm) on ln($c$)

| | Antibiotic | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Parameter | AMP | VAN | TET | NIS | SUB | GEN | KAN | ERY |
| $x^2$/ln($c$) model | | | | | | | | |
| MIC | 4.0 | 1.9 | 1.4 | 8.4 | 1.9 | 87 | 57 | 0.70 |
| ($R^2$) | 1.000 | 1.000 | 0.978 | 0.979 | 0.964 | 0.882 | 0.907 | 0.838 |
| $x$/ln($c$) model | | | | | | | | |
| MIC | 1.9 | 1.1 | 0.34 | 3.4 | 1.1 | 27 | 15 | 0.17 |
| ($R^2$) | 0.977 | 0.986 | 0.999 | 0.998 | 0.984 | 0.974 | 0.970 | 0.934 |

AMP, ampicillin; VAN, vancomycin; TET, tetracycline; NIS, nisin; SUB, subtilin A; GEN, gentamicin; KAN, kanamycin; ERY, erythromycin.
Average MIC and $R^2$ values from three repeats are presented.
The quadratic model is more accurate for vancomycin and ampicillin, whereas linear fits are more accurate in all other cases.



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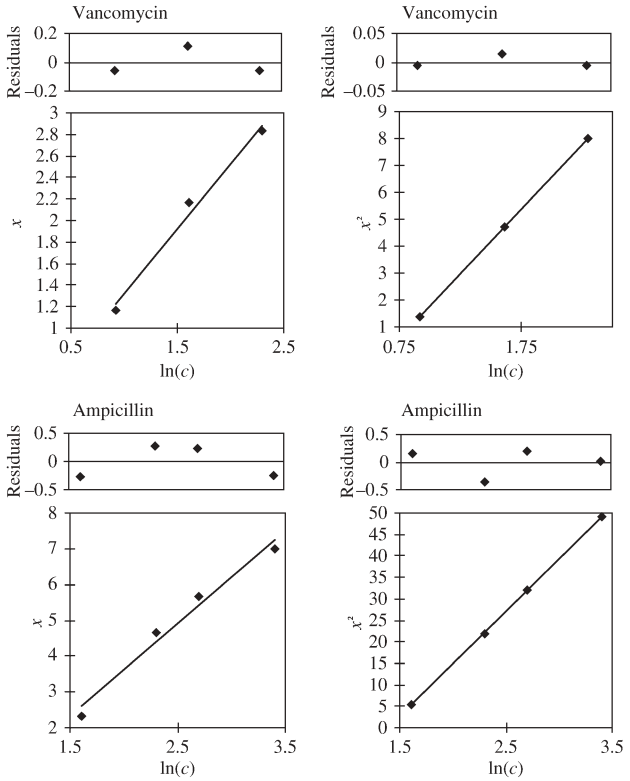


**Figure 2.** Agar diffusion of ampicillin and vancomycin; inhibition zone radii $x$ (mm) and their squared values $x^2$ are plotted against the logarithm of concentration together with linear fits. Residuals are shown above each plot. Free diffusion is described by $x^2$ versus ln($c$) plots and dissipative diffusion by $x$ versus ln($c$). Better linear fits are obtained using the free diffusion model. Zone sizes from a representative plate are shown.

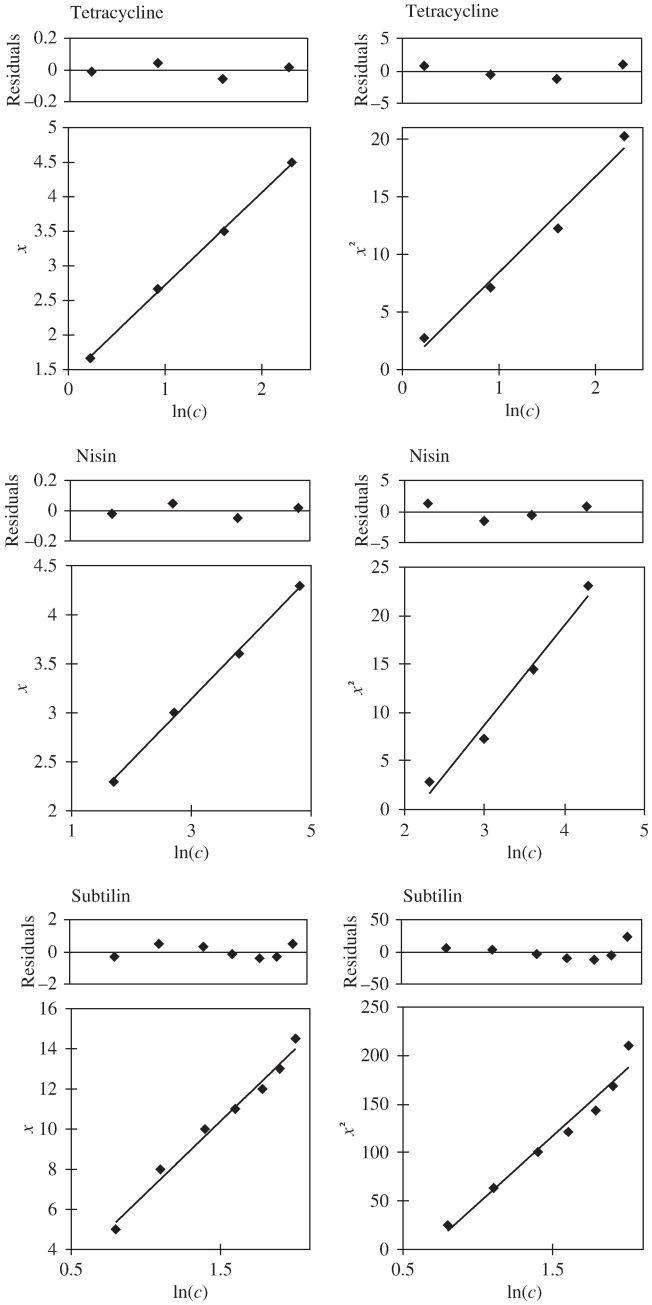


**Figure 3.** Agar diffusion of tetracycline, nisin and subtilin A; inhibition zone radii $x$ (mm) and their squared values $x^2$ are plotted against the logarithm of concentration together with linear fits. Residuals are shown above each plot. Free diffusion is described by $x^2$ versus ln($c$) plots and dissipative diffusion by $x$ versus ln($c$). Better linear fits are obtained using the dissipative diffusion model. Zone sizes from a representative plate are shown.

0.979 and 0.978 from the $x^2$/ln($c$) fit, respectively. The free diffusion model overestimates the MICs ~3-fold. Figure 4 summarizes diffusion results from gentamicin, kanamycin and erythromycin, which are better described by the linear, absorptive model but also show some monotonic non-linearity. Clearly, the free diffusion model gives inferior prediction of MIC with $R^2$ values ranging from 0.838 for erythromycin to 0.907 for kanamycin. The linear model gives a better prediction with $R^2$ values of 0.934–0.974, respectively. The determined MIC values are four times lower from the absorptive model (Table 1).

## Discussion

The agar diffusion assay is an important technique for assessing microbial susceptibility to antibiotics, which has found application worldwide over the past 50 years. It has a number of variation, which include the cup method,[4] the paper disc method,[16] the standardized single disc method,[11] as well as related approaches like the Etest (compare with Brown and Brown[17]). Determination of MIC using these approaches, as well as using the microdilution technique, has been shown to produce comparable results.[18]

A number of factors affect the accuracy and reproducibility of the agar diffusion method, including thickness and uniformity of the gel, the choice of cut-off size for the inhibition zones and breakpoints, temperature etc. When these factors are controlled or taken into consideration, analysis of data from the agar diffusion assays relies on theoretical models, which incorporate a number of important additional assumptions. It is important to understand these assumptions, which justify the use of these theoretical models and, at the same time, introduce some



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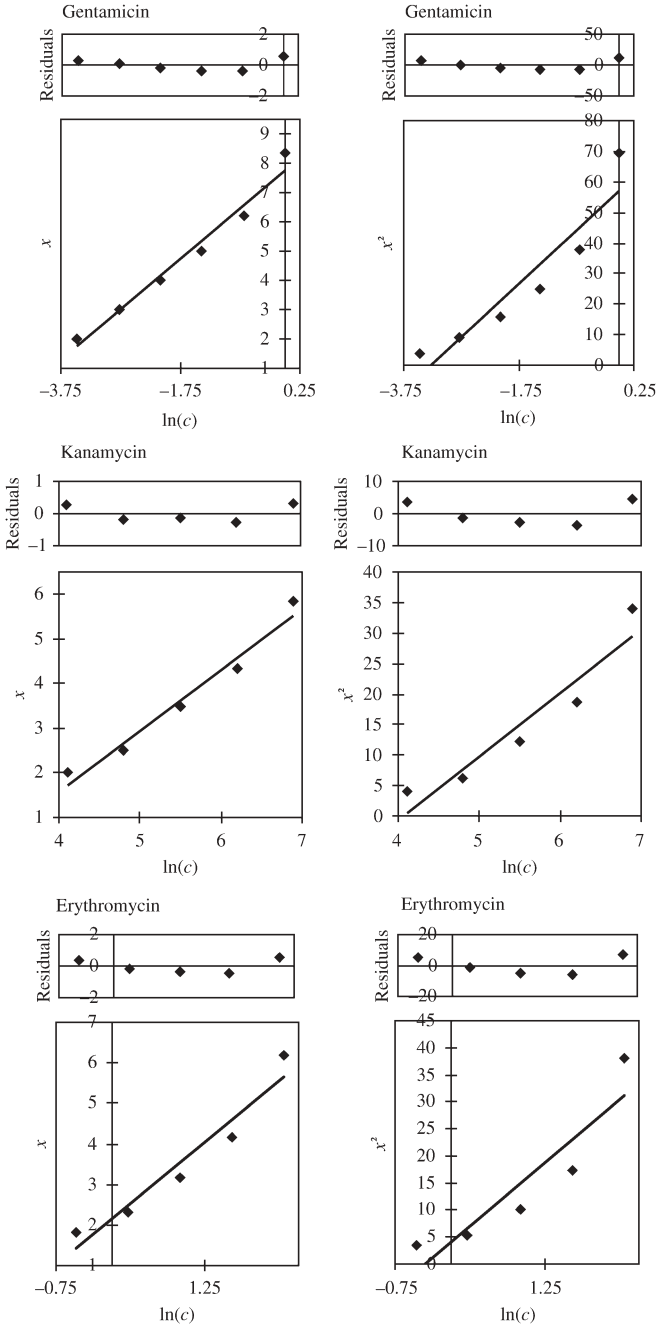


**Figure 4.** Agar diffusion of gentamicin, kanamycin and erythromycin; inhibition zone radii $x$ (mm) and their squared values $x^2$ are plotted against the logarithm of concentration together with linear fits. Residuals are shown above each plot. Free diffusion is described by $x^2$ versus ln($c$) plots and dissipative diffusion by $x$ versus ln($c$). Better linear fits are obtained using the dissipative diffusion model. Residual systematic deviation from linear dependence is observed, most noticeably for erythromycin. Zone sizes from a representative plate are shown.

limitations in the validity of each model. Theoretical analysis of antibiotic diffusion data by the disc method is built on the assumption that antibiotics diffuse freely and the diffusion-limiting factor is hydrodynamic viscous drag.[5] The most commonly used model is based on linear diffusion in a semi-infinite space[7] and is exemplified by the propagation of antibiotics in an agar-filled capillary.[7,19] Linear diffusion is described by equation (2) and MIC is determined using equation (1).[7,8,20] The use of this approach allows the accurate determination of susceptibility to penicillins and other antibiotics.[5,21] Our diffusion results for ampicillin and vancomycin fit well this model.

Often, the free linear diffusion model does not describe accurately the variation of inhibition zone size with antibiotic concentration. Notable examples include subtilin[9] and tetracycline,[22] where the dependence of zone size on the logarithm of concentration is linear, rather than quadratic. Deviations from the free diffusion model have prompted the development of other models, where the dependence of zone size on concentration is assumed to be described by a quadratic form[23] or where the two-dimensional nature of the problem is taken into account and solutions to the radial diffusion equation are used.[24] The former approach is simple and fairly accurate over a small range of concentrations, whereas the latter describes well zone size over a fairly broad range of concentrations but the solutions are complex, including infinite series of special functions, the use of which in routine microbiological work is impractical.

The new model for describing diffusion in agar, proposed here, is built on the assumption that during the diffusion process, part of the antibiotic is lost either through interactions with the solid component of the medium, aggregation or through another mechanism of inactivation. We discuss the equation for linear diffusion only [equation (4)] and its solution [equation (3)] as they offer a simple and practical tool for analysis of diffusion data. The solutions of the dissipative radial diffusion equation resemble in their form those from the free diffusion case[24] and involve the use of special functions. They are not presented here, as the dependence of zone diameter on logarithm of concentration is well described by either the free or the dissipative models in most cases of practical importance and the complex mathematical treatment offers little additional benefit.

Analysis of our experiments with diffusion in agar plates showed that inhibition zone sizes from vancomycin and ampicillin are described well by the free linear diffusion model. However, the dependence of zone size on logarithm of concentration from all other antibiotics tested was best described by a linear function of ln($c$), rather than a quadratic. Two groups emerged—zone sizes from nisin, subtilin and tetracycline fitted best the linear model, whereas gentamicin, kanamycin and erythromycin showed some residual deviation from our model. The concentration dependence within the latter group was even weaker than linear, which may indicate the existence of a range of susceptibility of the test organism to these antibiotics, rather than a single cut-off concentration.

Another possible explanation requires considering the mode of antibiotic action. In the first group (Figure 3), tetracycline is internalized by bacteria using an active transport mechanism. Nisin and subtilin act on the outer leaflet of the bacterial plasma membrane in a pyrophosphate[13,25] and lipid II-dependent[26] manner and the subsequent metabolic deregulation[27] results from membrane breach. In contrast, the antibiotics from the second group, gentamicin, kanamycin and erythromycin (Figure 4), must cross the intact bacterial membrane in order to reach their target sites. Consequently, the antibiotic concentration at the target depends on a number of factors, possibly including multidrug pumps, and may not reflect accurately the antibiotic



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concentration in the medium, described by any of the existing diffusion models. While such deviation is important, it appears to be secondary in magnitude to the effect of antibiotic dissipation in the medium, described in this text.

A number of factors may affect the accuracy of the agar diffusion assays. The assays are usually carried out using multiple discs on the same Petri dish to eliminate differential effects from growth time and temperature. Caution is required during preparation for the assay, as agar homogeneity and thickness, as well as other factors can affect zone size and shape.[28] One particular case for the use of agar diffusion tests instead of broth dilution assays is their robustness in studies of surface active antibiotics, for example nisin or subtilin.[13] In broth dilution assays, bacterial susceptibility depends on the surface concentration of antibiotic, which partitions preferentially onto the bacterial membrane and makes inhibition critically dependent on the ratio of antibiotic solution concentration to inoculum size. Agar diffusion methods do not expose the test bacteria to the full volume of antibiotic solution and are less sensitive to the size of the inoculum.

### *Conclusions*

We propose a new approach to the analysis of agar diffusion data, in which we assume dissipation of the diffusing agent during its propagation through the agar medium. This model predicts a linear dependence of indicator zone sizes on the logarithm of concentration of the diffusing agent. It provides validation for analysis of diffusion data by others, who have observed such linear dependence. MICs can be obtained with accuracy from a number of antibiotics using simple linear regression analysis. The applicability of this model and the widely used free diffusion model was tested on a range of antibiotics and we conclude that the validity of each method should be tested on the individual compound and can depend on the nature on the antibiotic, among other factors. Diffusion of ampicillin and vancomycin was better described by the existing, free diffusion model, whereas diffusion of tetracycline, nisin, subtilin, gentamicin, kanamycin and erythromycin was more accurately analysed by the dissipative diffusion method. MICs, determined by the two methods, differed by a factor of two to three and were determined with higher accuracy by one of the methods. The method proposed here, in combination with the existing approach, provides greater accuracy of the agar diffusion technique for a range of antibiotics and antimicrobial peptides.[29] A simple web tool, which allows a quick determination of MIC by the most suitable method, is currently being developed.

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## Transparency declarations

None to declare.

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