ELSEVIER

Journal of Microbiological Methods 42 (2000) 159–165

Journal of Microbiological Methods

www.elsevier.com/locate/jmicmeth

A sensitive standardised micro-gel well diffusion assay for the determination of antimicrobial activity

E.A. du Toit, M. Rautenbach*

Department of Biochemistry, University of Stellenbosch, Private bag X1, Matieland, Stellenbosch 7602, South Africa

Received 15 February 2000; received in revised form 10 June 2000; accepted 20 June 2000

Abstract

We have developed a highly sensitive micro-gel well diffusion assay for the determination of antimicrobial activity. In essence, the normal radial diffusion type assay was adapted to perform it in a microtiter plate. We compared our micro-gel well diffusion assay to a radial diffusion assay and a microtiter broth dilution method, using gramicidin S as model antibiotic, and *Micrococcus luteus* as the indicator organism. The micro-gel well diffusion assay was as sensitive as the microtiter broth dilution method, and approximately twice as sensitive as the radial diffusion method. Data analysis to calculate minimum inhibitory concentration, 50% microbial growth inhibition and maximum inhibitory concentration was refined by generating dose–response curves with the software package Prism® 3.0 (Graphpad Software Inc.). The minimum inhibitory concentrations, determined by the three methods, were significantly different ($P < 0.001$), highlighting the limitations involved in comparing data obtained from different methods.

Keywords: Low-electroendosmosis-type agarose; Antimicrobial activity assay; Minimum inhibitory concentration; 50% microbial growth inhibition; Maximum inhibitory concentration

1. Introduction

With the discovery of antibiotics and vast numbers of antimicrobial peptides, numerous methods have been developed to test these compounds against various organisms. In the beginning, for lack of alternatives, bacteria were incubated with these compounds and the rate of decrease in viable counts monitored. This, however, was found to be too time and material consuming, and new, more efficient methods were sought.

A number of methods were since developed with the emphasis on the conservation of test material. The earliest, most successful methods simply entailed the formation of inhibition zones by an antibiotic in a lawn of bacteria growing in agar (Barry, 1980). The inhibition zones were physically measured to determine the antimicrobial activity of the antibiotic. For example, the agar cup assay method for determining the activity of penicillin was developed as far back as 1946 (Cooper and Woodman, 1946). Researchers favoured these methods because they required very little test material and could be carried out relatively easily. Later, however, agar was found to be unsuitable for testing certain antimicrobial molecules, as it contained specific extrinsic substances or contaminants that hampered the diffusion of these molecules. This led to agar

*Corresponding author. Tel.: +27-21-808-5872; fax: +27-21-808-5863.

E-mail address: mra@maties.sun.ac.za (M. Rautenbach).

0167-7012/00/$ – see front matter
PII: S0167-7012(00)00184-6

being replaced by a high quality agarose in many of the assays (Kunin and Edmondsen, 1968).

To date the microtiter plate has played an integral role in the development of various micro-assays, with one of the most well-known being the enzyme-linked immunosorbent assay (ELISA) technique (Engvall, 1980). Literally hundreds of assays are now performed in microtiter plates, because 96 wells can be used per experiment, small amounts of test material are required, and results can easily be obtained spectrophotometrically using a microtiter plate reader.

We developed a highly sensitive micro-gel well diffusion assay by adapting an inhibition zone assay to perform it in a microtiter plate. In this paper our method is compared to an inhibition zone assay and a microtiter broth dilution method. Gramicidin S (an antibiotic cyclodecapeptide from *Bacillus brevis*) was used as the model antibiotic and *Micrococcus luteus* (a Gram-positive bacterium) as the indicator organism.

2. Materials

Micrococcus luteus (NCTC 8340) was used as indicator organism in all experiments. Dr. R. Levitt, from Fine Chemicals, South Africa, donated synthetic gramicidin S. Microtiter plates (Nunc-Immuno™ Maxisorp) were from Nalge NUNC International (Roskilde, Denmark), Falcon® tubes from Becton Dickinson Labware (Lincoln Park, USA), and culture dishes from Quality Scientific Plastics, USA. Low-electroendosmosis-type agarose (D1-LE) was from Whitehead Scientific (Brackenfell, South Africa). Tween 20 and Coomassie brilliant blue R-250 were from Fluka (Buchs, Switzerland). Dulbecco's phosphate buffered saline (PBS) was either from Life Technologies (Faisley, Scotland), or prepared in the laboratory (Dulbecco and Vogt, 1954). Sodium chloride and glacial acetic acid were from Saarchem (Krugersdorp, South Africa). Disodium hydrogen phosphate, methanol (99%), and formaldehyde (99%) were from Merck (Darmstadt, Germany). Tryptone soy broth (TSB; soybean-casein digest medium USP, pH 7.3 ± 0.1) was manufactured by Biolab Diagnostics (Midrand, South Africa). Potassium chloride, potassium dihydrogen phosphate, dimethylsulphoxide (DMSO) and casein were from Merck (Midrand, South Africa). Bovine serum albumin (BSA) was from Boehringer Mannheim (Germany). Analytical quality water was prepared by filtering glass distilled water through a Millipore Milli Q® water purification system.

3. Methods

3.1. Preparation of cells

3.1.1. Radial diffusion assay (RDA) and the micro-gel well diffusion assay

Bacteria were grown overnight at 37°C in TSB, sub-cultured and grown to an optical density (OD) of 0.6 at 620 nm. The cells were centrifuged for 10 min at $900 \times g$. The supernatant was discarded and the cells were washed once with 10 ml cold Dulbecco's PBS by centrifugation. Finally, the cells were diluted to an OD of 0.6 in Dulbecco's PBS (Lehrer et al., 1991).

3.1.2. Microtiter broth dilution method

Bacteria were grown overnight at 37°C in TSB, sub-cultured and grown to an OD of 0.6 at 620 nm. The cells were diluted with TSB to 5×10^5 colony forming units per ml (CFU/ml) based on the relationship OD_{620} $0.20 = 5 \times 10^5$ CFU/ml (Lehrer et al., 1991).

3.2. Radial diffusion assay

The radial diffusion assay was adapted from the method of Lehrer et al. (1991). A gel solution containing 1% (w/v) of powdered TSB medium, 1% w/v of low-electroendosmosis-type agarose, and 0.02% (v/v) Tween 20 made up in Dulbecco's PBS was prepared and autoclaved. Culture dishes were blocked with 0.5% casein in Dulbecco's PBS for 1 h and dried under ultraviolet light for a further 4 h. Ten ml of the gel, at 45°C, was aliquoted and added to 1 ml of the dilute bacterial culture and dispersed for 10 s using a laboratory vortex. Once the bacteria were adequately dispersed, the gel was poured into a circular culture dish on a level platform. The gel was then allowed to set for 1 h, before wells were made using a 5 mm punch. The test sample was dissolved

in analytical quality water and then further diluted (doubling dilutions). After adding 15 μl of sample material to each well, the plates were incubated for 3 h at 37°C and then turned over and incubated for a further 14 h at 37°C. The gel was stained for 24 h in a dilute solution of Coomassie brilliant blue R-250 (2 mg dye; 27 ml methanol; 63 ml water and 15 ml 37% formaldehyde). The spent staining solution was decanted, replaced with an aqueous solution of 10% acetic acid and 2% DMSO and left for ~10 min. The areas of the clear zones surrounding the wells were calculated from calliper measurements of a 190% enlarged photocopy of the stained gels. The gels were stored in water at 4°C.

3.3. Microtiter broth dilution method

The microtiter broth dilution method, microbroth dilution assay, and versions of it have previously been described by a number of investigators (Amsterdam, 1996; Steinberg and Lehrer, 1997; Hancock, 1997). The test sample was dissolved in analytical quality water at twice the required maximal concentration, and then diluted 1:1 with 0.02% acetic acid, 0.4% bovine serum albumin (BSA) to the required stock concentration. Further doubling dilutions were made using 0.01% acetic acid, 0.2% BSA. TSB (100 μl) was pipetted into each of the wells of the first column of the microtiter plate as a sterility control and blank. The rest of the wells each received 100 μl of the bacterial suspension. A volume of 30 μl of the test sample was pipetted into all wells excluding the first two rows that served as growth controls. The plate was covered and incubated at 37°C for 17 h and the light dispersion in each well determined using a microtiter plate reader (Multiscan Titertek) at 620 nm.

3.4. Micro-gel well diffusion assay

Microtiter plates were blocked with 0.5% casein in Dulbecco's PBS for 1 h and sterilised under ultraviolet light for a further 4 h. The gel was prepared as in the radial diffusion assay and kept in a water bath at 46±1°C to maintain its temperature at 45±1°C. The following modified pipetting technique was essential for obtaining reproducible results: 70 μl of the heated gel suspension were drawn up, using a Gilson® micropipette, by depressing the pipette plunger to its maximum, and pipetted by depressing the plunger only as far as its first stoppage point. The normal method of pipetting was therefore reversed to prevent bubble formation. The first column of the microtiter plate received only 70 μl of gel, to serve as blank and sterility control. The other wells each received 70 μl of a bacterial–gel suspension (70 μl bacteria and 700 μl gel dispersed with a laboratory vortex). The suspension was prepared for each column immediately before application. Once the desired number of wells were prepared, the gel was allowed to set for 30 min. The test sample was dissolved in analytical grade water and then further diluted (doubling dilutions). After applying 30 μl of sample directly onto the gel in the wells the microtiter plate was covered and incubated for 17 h at 37°C. The light dispersion per well was determined using a Multiscan Titertek microtiter plate reader at 620 nm. The plates were preserved by adding 140 μl of a solution containing 27 ml methanol, 63 ml water and 15 ml 37% formaldehyde to each well. The gel was stained by adding 100 μl of a 0.002% solution of Coomassie brilliant blue R-250. After 24 h the colouring solution was discarded and replaced by water before storage at 4°C.

3.5. Data processing

All data were analysed using Graphpad Prism version 3.0 for Windows, GraphPad Software, San Diego, CA, USA (www.graphpad.com).

Nonlinear regression was performed on the dose–response data and a sigmoidal curve with variable slope was fitted to each of the data sets.

The equation used for the sigmoidal curve with variable slope was:

$$Y = \text{bottom} + (\text{top} - \text{bottom})/\left(1 + 10^{\log \text{IC}_{50} \times \text{Hill slope}}\right)$$

where bottom is the *Y*-value at the bottom plateau; top is the *Y*-value at the top plateau; log IC_{50} is the *X*-value of response halfway between top and bottom; Hill slope is the Hill coefficient or slope factor (controls slope of curve). For curve fitting only the mean value of each data point, without weighting, was considered. The minimum inhibitory concentration (MIC) and the maximal inhibitory concentration (IC_{max}) were calculated from the *x*-values at

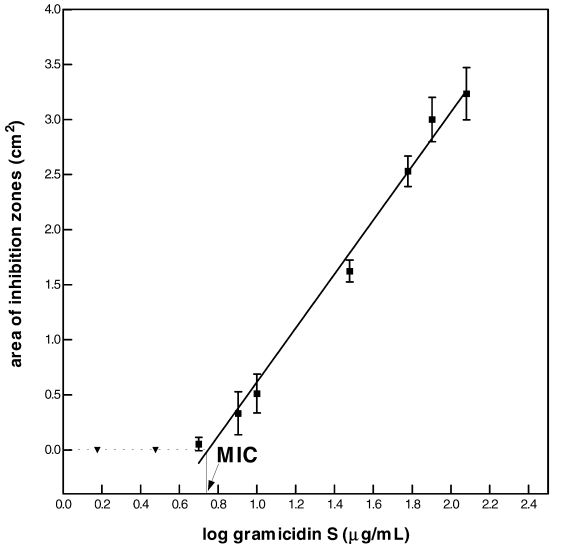

Fig. 1. An example of a dose–response curve from a RDA experiment. The solid line indicates a linear response ($r^2 = 0.99$) over the concentration range: 5 to 120 μg/ml. The dashed line indicates the concentration range where no inhibition zones were visible (<3 μg/ml). The MIC value shown on the graph is 5.4 μg/ml. The error bars represent the S.E. for each concentration value (quadruplicate determinations).

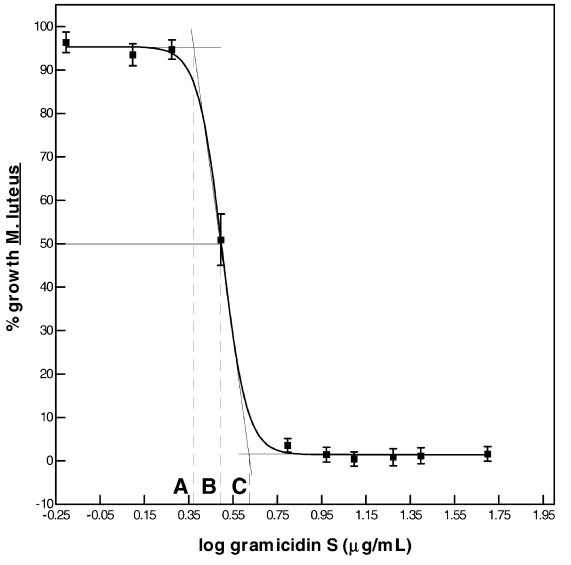

Fig. 2. An example of a sigmoidal dose–response curve from a microtiter broth dilution method experiment. The MIC value (1.6 μg/ml), the IC_{50} value (2.2 μg/ml) and the IC_{max} value (2.7 μg/ml) were calculated from the *x*-values indicated at A, B, and C respectively. The error bars represent the S.E. for each concentration value (quadruplicate determinations).

the intercept between the slope and the bottom plateau and top plateau, respectively. The 50% inhibitory concentration (IC_{50}) was calculated from the *x*-value of the response halfway between top and bottom plateau. Also refer to Figs. 1–3.

A statistical analysis of the results was done using the statistical functions of the Graphpad Prism 3.0 software. MIC values, obtained from all three methods, were analysed doing a one-way analysis of variance (one-way ANOVA). IC_{max} and IC_{50} values obtained from the microtiter broth dilution method and the micro-gel well diffusion method were analysed statistically using the unpaired *t*-test.

4. Results and discussion

Our decision to use *M. luteus* as indicator organism was based upon the need to develop a sensitive assay for a cyclic antimicrobial peptide (iturin A) with *M. luteus* as its only bacterial target. Gramicidin S was a logic choice as model antibiotic due to its availability and analogous cyclic structure. We found

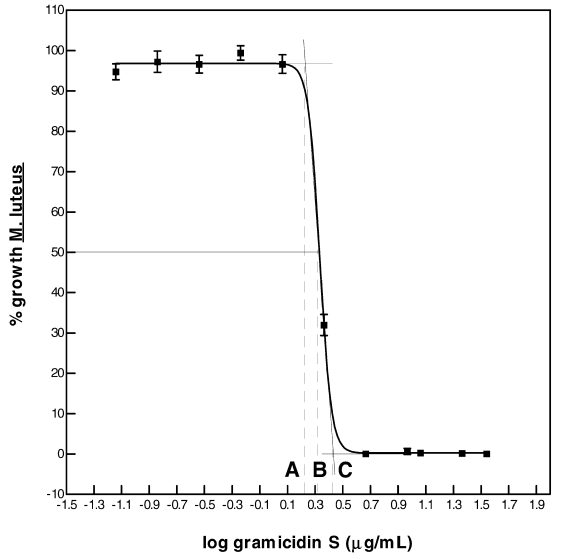

Fig. 3. An example of a sigmoidal dose–response curve from a micro-gel well diffusion assay experiment. The MIC value (2.3 μg/ml), the IC_{50} value (3.2 μg/ml), and the IC_{max} value (4.3 μg/ml) were calculated from the *x*-values indicated at A, B, and C, respectively. The error bars represent the S.E. for each concentration value (quadruplicate determinations).

that all three methods under investigation were compatible with the model antibiotic, gramicidin S, and with the indicator organism, *M. luteus*. The three methodologies are compared in Table 1.

Experiments using RDA provided highly visual results. Sharply defined zones of clearing could be observed as a consequence of the growth inhibition of *M. luteus* by various concentrations of gramicidin S. The inhibition zone areas were found to be directly proportional ($r^2 = 0.99$) to the concentration of gramicidin S over a concentration range from 5 to 120 μg/ml (Fig. 2). From a concentration of 3.0 μg/ml and less no further inhibition zones were observed (Fig. 1). From these results we obtained a MIC value of 5.3±0.1 μg/ml for gramicidin S.

Sigmoidal dose–response curves were obtained after analysis of the results from the microtiter broth dilution method and the micro-gel well diffusion assay. From these curves the MIC, IC_{50}, and IC_{max} values were deduced (Figs. 2 and 3, Table 2). The growth curves for the bactericidal gramicidin S remained sigmoidal over time (17–24 h), but with the slopes increasing (results not shown). The MIC and IC_{50} values consequently increased with time, but the IC_{max} value remained relatively constant.

A comparison of the MIC values from the two types of microtiter-based assays showed them to be approximately twice as sensitive as the RDA (2.3, 2.0 μg/ml vs. 5.3 μg/ml). Furthermore, all three MIC values were significantly different ($P < 0.001$). The IC_{max} and IC_{50} values from the micro-gel well diffusion assay and microtiter broth dilution method were also significantly different, with $P < 0.05$. The observed variance between the results from the two microtiter-based assays could be attributed to differences in both growth conditions (broth vs. gel) and initial cell densities. Staining the gel in the micro-gel well diffusion method notably enhanced the visible inhibition in each well. This created an excellent visual record of the result (Fig. 4). We found,

Table 1
A comparison of the protocols for the RDA, micro-gel well diffusion assay, and microtiter broth dilution method

	Radial diffusion assay	Micro-gel well diffusion assay	Microtiter broth dilution method
Experimental container	Petri dish	Microtiter plate	Microtiter plate
Prevention of peptide–plastic interaction	Coated with casein buffer	Coated with casein buffer	Sample in 0.01% acetic acid, 0.2% BSA
Growth medium	Low-endosmosis type agarose + TSB	Low-endosmosis type agarose + TSB	Tryptone soy broth
Cell count	1.4×10^7 CFU/ml	7.5×10^5 CFU/ml	5×10^5 CFU/ml
Incubation time	17 h	17 h	17 h
Measurement method	190% enlargement of inhibition zones	Light dispersion at 620 nm	Light dispersion at 620 nm

Table 2
MIC, IC_{50}, and IC_{max} values of gramicidin S against *M. luteus*. Three independent experiments were performed per method and inhibition at each concentration was determined in quadruplicate per experiment

Method	MIC (μg/ml)[a,b]	IC_{50} (μg/ml)[a,c]	IC_{max} (μg/ml)[a,c]
Micro-gel well diffusion assay	2.3±0.1	3.4±0.1	4.8±0.2
Microtiter broth dilution method	1.9±0.1	2.2±0.1	2.6±0.1
Radial diffusion assay	5.3±0.1	NA	NA

[a] Values are means±standard error of the mean (S.E.).
[b] MIC values from all three methods differed significantly from each other ($P < 0.001$).
[c] IC_{max} and IC_{50} values from the microtiter broth dilution method and micro-gel well diffusion assay were significantly different ($P < 0.05$).

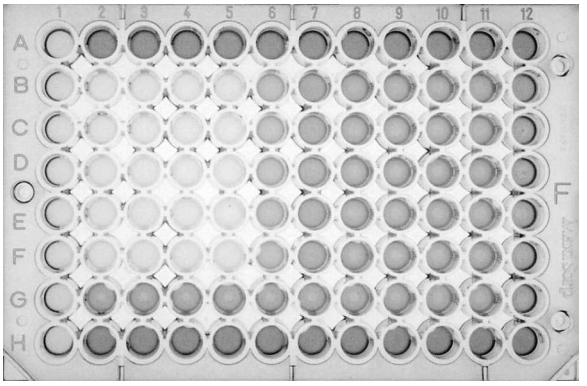

Fig. 4. A photograph of a micro-gel well diffusion assay experiment after fixation and colouring. Column one contains only gel to serve as a blank and sterility control. Rows A, G, and H, starting at column two, contain the *M. luteus*–gel suspension without gramicidin S. Wells B2 to F2 contain a bacterial–gel suspension with gramicidin S (200 μg/ml), from where consecutive columns contain doubling dilutions of the peptide. The final column (wells B12 to F12) contains the lowest gramicidin S concentration (0.2 μg/ml).

however, that staining did not enhance the sensitivity, and it is recommended that this be done after the plate has been scanned on the microtiter plate reader.

Comparative assays of the RDA and micro-gel well diffusion assay showed that blocking the plate with casein did not affect the IC_{50} values, and that sterility was also not compromised (results not shown). Even though the IC_{50} value remained unchanged with gramicidin S as test compound, this might not be the case for all antimicrobial compounds, therefore this blocking step is recommended.

5. Conclusions

In general, all three methods were easily manageable and highly repeatable results were obtained. There are, however, some advantages and disadvantages to each of the methods. The use of a microtiter plate allows for a much larger and more economical experiment, as opposed to a culture dish which can accommodate far fewer wells per dish. Furthermore, determining inhibition by a spectrophotometric method, using a microtiter plate reader, is more sensitive and accurate than physically measuring zones of inhibition, because of possible human bias in measuring. Regarding the methodologies of the microtiter broth dilution method and the micro-gel well diffusion assay, it is clear that the microtiter broth dilution method is the less labour intensive of the two, and is therefore ideal for screening large numbers of test samples. The use of a liquid broth as growth medium also allows for almost immediate antibiotic–cell interaction, as opposed to agar or agarose where it is depends on diffusion rate. Agar or agarose, on the other hand, is advantageous in that microbial heterogeneity or contamination can be detected readily by observing the nature of bacterial growth, as opposed to a broth where a contamination would not be directly discovered (Barry, 1980). Results from the agarose-based assays were also easily manipulated through the use of fixing agents and staining agents. By using a fixing agent it is possible to halt the experiment at any specific time, as well as to preserve the experiment for an undetermined period of time. By using a staining agent it is possible to create a visual record of the result, but also can be very informative depending on the type of staining used.

The RDA gave a good indication of antimicrobial activity through the formation of inhibition zones. This method, however, was not as sensitive as the other two. No inhibition zones were visible at gramicidin S concentrations <3.0 μg/ml. This result could possibly be attributed to the fact that at very low concentrations, the test sample might not diffuse far enough into the gel to form a visible inhibition zone. The micro-gel well diffusion assay, in contrast, only depends upon the localised downward diffusion of the test sample. Because the reaction area is not as large as that of the RDA, diffusion of molecules of varying molecular size is also no longer a limiting factor.

In other experiments in our laboratory, the versatility of the micro-gel well diffusion assay was demonstrated by using various other antimicrobial compounds, as well as *Escherichia coli* as Gram-negative indicator organism. Antimicrobial compounds assayed included the cationic frog peptide, magainin 2 and some N-terminal deletion analogues, cationic model peptides from a combinatorial library, and the antibiotic tetracycline.

Generally, there is some difficulty in standardising antimicrobial assays because there is no single assay

that is compatible with all antimicrobial compounds. It seems therefore that researchers will have to decide upon, and make use of a method best suited to their work. From the statistical analysis of the results from the three methods, it was shown that all three sets of results differed significantly from each another. This emphasises the fact that if identical assays are not used, it will not be feasible to compare results. The micro-gel well diffusion assay and our method of data analysis, using Graphpad Prism 3.0 software, improved the sensitivity of the current agar or agarose-based assays.

6. Notation

BSA	bovine serum albumin
CFU/ml	colony forming units per millilitre
DMSO	dimethylsulphoxide
ELISA	enzyme-linked immunosorbent assay
IC_{50}	50% inhibitory concentration
IC_{max}	maximal inhibitory concentration
MIC	minimum inhibitory concentration
OD	optical density
PBS	phosphate buffered saline
RDA	radial diffusion assay
S.E.	standard error of the mean
TSB	tryptone soy broth

References

Amsterdam, D., 1996. Susceptibility testing of antimicrobials in liquid media. In: Lorian, V. (Ed.), Antibiotics in Laboratory Medicine. Williams and Wilkins, Baltimore, MD, pp. 52–111.

Barry, A.L., 1980. Procedure for testing antibiotics in agar media: theoretical considerations. In: Lorian, V. (Ed.), Antibiotics in Laboratory Medicine. Williams and Wilkins, Baltimore, MD, p. 1.

Cooper, K.E., Woodman, D., 1946. The diffusion of antiseptics through agar gels, with special reference to the agar cup assay method of estimating the activity of penicillin. J. Pathol. Bacteriol. 58, 75.

Dulbecco, R., Vogt, M., 1954. Plaque formation and isolation of pure lines with Poliomyelitis viruses. J. Exp. Med. 98, 167.

Engvall, E., 1980. Enzyme Immunoassay ELISA and EMIT. In: van Vunakis, H., Langone, J.J. (Eds.). Methods in Enzymology, Vol. 70. Academic Press, New York, pp. 419–439.

Hancock, R.E.W., 1997. Hancock Laboratory Methods, www.interchg.ubc.ca/bobh/peptides.htm.

Kunin, C.M., Edmondsen, W.P., 1968. Inhibitor of antibiotics in bacteriological agar. Proc. Soc. Exp. Biol. Med. 129, 118.

Lehrer, R.I., Rosenman, M., Harwig, S.S.S.L., Jackson, R., Eisenhauer, P., 1991. Ultrasensitive assays for endogenous antimicrobial activity. J. Immunol. Methods 137, 167–173.

Steinberg, D., Lehrer, R.I., 1997. Designer assays for antimicrobial peptides: disputing the ‘one size fits all’ theory. In: Shafer, W.M. (Ed.), Methods in Microbiology. Humana Press, Totowa, NJ, pp. 169–187.