Development of quantitative methods of measuring antifungal drug effects in the rabbit cornea

ERASMUS O. OJI

From the Department of Ophthalmology, University Teaching Hospital, Jos, Plateau State, Nigeria

SUMMARY By means of multiple inoculation in each cornea with microtrephination a highly reproducible quantitative model of fungal infection of the rabbit corneal stroma has been produced. A known suspension of the chosen pathogen was systematically implanted into the trephine sites in the cornea. The degree of infectivity was monitored in both the preinoculation treated corneae (prophylaxis) and the postinoculation treated corneae (therapy). Examples measuring and comparing the antifungal effect of various imidazole drugs against Candida albicans are discussed.

The lack of a finely quantitated and highly reproducible method of infecting the cornea with pathogenic fungi has made existing animal models of keratomycosis cumbersome and imprecise for measuring the efficiency of antifungal drugs.

Burda and Fisher injected aspergillus and fusarium species intracorneally in rabbits, employing a tuberculin syringe equipped with a 25-30 gauge needle. Singer and Lawton-Smith injected Histoplasma capsulatum intracorneally by the same method but also scratched the corneal epithelium of their rabbits and inoculated the resulting lesions by dropwise application of H. capsulatum. Ishibashi injected Fusarium solani into various Japanese strains of rabbit to produce a workable model of keratomycosis.

O'Day et al. injected Fusarium solani into the anterior chamber of outbred Wistar rats for the study of oculomycosis, while Fine and Zimmerman studied the ocular effectiveness of nystatin by intravitreal injection of Aspergillus fumigatus in albino rabbits.

None of these methods is sensitive enough for accurate study of quantitative drug effects on the eye in reasonable numbers of animals. Quantitated methods of multiple infection of the rabbit or guinea-pig cornea have been in use in Moorfields Eye Hospital for many years and have enabled us to measure antiviral effects in vivo, at first by calculating and comparing infectivity titres and more recently by a method of corneal epithelial lesion inhibition assay that resembles, in principle, an in-vitro viral plaque reduction assay. The technique has further been developed by Shiota to provide a corneal epithelial lesion reduction assay that can measure therapeutic effects of antiviral drugs on established viral lesions.

The present paper reports the application of these principles to provide finely quantitated methods of measuring both prophylactic and therapeutic antifungal effects in the rabbit cornea.

Material and methods

INFECTIVE INOCULUM Two isolates of Candida albicans were investigated. The first was isolated from the human eye (M1475), while the second was isolated from the human skin (R1646). Each isolate was cultured on Sabouraud's dextrose agar for 4 days at 37°C, then washed off the medium with sterile saline, and diluted to give a normal concentration (N) of 1 million yeast particles per ml of suspension, from which serial 10-fold dilutions were prepared.

CORNEAL MICROTREPHINATION Sterile glass trephines 100 mm long and 1.5 mm diameter (Fig. 1) were used to cut 17 wounds, half corneal stromal thickness, in each of the rabbits' corneae under 10 times magnification of the Zeiss OpM1 operating microscope. New Zealand white rabbits weighing 2-3 kg were used. Fig 2 illustrates the configuration of the corneal microtrephinations. The central trephine cut is made at the apex of the cornea and serves as a guide for making 4 trephinations in each axis at 12-6, 2-8, 3-9, and 4-10 o'clock. It is very easy to perforate the cornea, so considerable practice and care are
Finally, when the infective titres to produce corneal infective doses 50% and 100% (CID_{50} and CID_{100}) were obtained, these suspensions were used to inoculate all 17 corneal wound sites in other rabbits to check for reproducibility of their respective infectivities in 6 rabbits (12 eyes).

Each pathogen suspension was stirred thoroughly before any inoculum was removed. Sterile blotting paper cut into small triangles were used to mop up any spillage from one site before it contaminated other trephine sites. Readings were made at 24 and 90

**Implantation of Infective Inocula**

The trephine tube used for microtrephination was dipped into the pool of chosen pathogen suspension containing 1 million particles per ml. Capillary action drew up about 10 mm of inoculum into the trephine tube. It was not necessary to plug the upper end of the tube. This loaded trephine tube was then inserted in each of the trephine sites in the 12–6 axis and gently rotated for 30 seconds, with the superior and inferior recti muscles as fixation points at 12 and 6 o’clock respectively.

Similarly, the pathogen suspension containing 10^6 particles per ml was inoculated in the 2–8 axis and the 10^4 and 10^3 suspensions in the 3–9 and 4–10 axes respectively (Fig. 3).

![Sterile glass trephines, 100 mm long and 1.5 mm in diameter.](image)

![Pattern of inoculation of serial dilutions of Candida albicans on the microtrephine sites on the rabbit's cornea.](image)

![Configuration of 17 corneal microtrephinations on each cornea of the New Zealand white rabbit.](image)
Development of quantitative methods of measuring antifungal drug effects in the rabbit cornea

48 hours after corneal inoculation with pathogens, the animals being under general anaesthesia.

**Anaesthesia and Analgesia**
Microtrephination, implantation of the infective pathogens, and monitoring the results were all performed under general anaesthesia with either sodium pentobarbitone (Sagatal) or alphaxalone and alphadalone (Althesin) given by very slow intravenous injection. These anaesthetic agents provide minimal analgesia, which may be antianalgesic, so a retrobulbar injection of a local anaesthetic (0.5 ml-0.9 ml of 2% lignocaine) was usually given for analgesia and absolute ocular akinesia.

**Calculation of 50% Corneal Infective Dose**
Each circle of infection at the trephine sites was divided into 4 quadrants. Fig. 4 illustrates diagramatically the method of scoring the lesions. The apical wound in the 1:10 serial dilution experiments was used as a guide in the microtrephination and was not counted in scoring in these initial experiments. However, when the \( CID_{50} \) and \( CID_{100} \) of the pathogens had been established, all the 17 trephine sites were counted. The percentage infectivity was calculated as:

\[
\text{Number of quadrants counted as infected (Y)} \times \frac{100}{\text{Maximum number of quadrants which can be infected (68)}}
\]

i.e., \( \frac{Y}{68} \times \frac{100}{1} = \text{infectivity} (68 = 17 \times 4) \)

50% infectivity = \( CID_{50} \)
100% infectivity = \( CID_{100} \)

Figs. 5a and 5b illustrate diagramatically a 65% and a 100% corneal lesion scored by the above method.

**Drug Prophylaxis and Therapy**
The chosen drug (1% clotrimazole or 1% miconazole) dissolved in arachis oil was used to treat prophylactically one of the eyes twice for 2 consecutive hours. The treated eyes were then left for a period of 2 hours before inoculating them with the pathogenic suspension of the *Candida albicans* (R1646). The control eyes were treated with the vehicle alone (arachis oil). The eyes for treatment and for control in each animal group were randomly allocated.

When 100% corneal infectivity had been estab-
lished, the eyes (both control and test) were treated hourly for at least 6 to 10 consecutive hours daily until there was complete resolution of the corneal lesions.

**Histopathology**

Corneal and conjunctival swab specimens were taken from both test and control eyes for mycological and bacteriological studies daily after inoculation till sterile results were obtained.

Some of the test and control eyes were enucleated at different stages of the infective process in the cornea for histopathological studies.

Statistical analysis of the results was by simple paired *t* test, confirmed by computer analysis of variance.

**Results**

Table 1 shows the infectivity rates of various inoculum suspensions in 4 rabbits’ cornea for the 2 candida isolates (M1475) and (R1646), by the method of corneal microtrephination and inoculation of identical sites with serial dilutions of the 2 isolates of Candida albicans as illustrated in Fig. 3.

Readings at 48 hours showed a gradation of infectivity from the lower suspensions to the higher suspensions of the pathogens.

In all 4 rabbits in this group the isolate (R1646) produced higher infectivity rates in all the suspensions studied when compared with the isolate (M1475). The former was therefore chosen for further studies.

Table 2 shows the infectivity rates on 6 rabbit corneas (12 eyes). The 10⁴ dilution caused a mean infectivity of 61.9% 48 hours after inoculation, while the 10⁴ dilution gave a mean infectivity of 38.9% after 48 hours. From these results it was calculated that a dilution 10⁻³ would give a 50% corneal infective dose, CID₅₀.

Table 3 shows the infectivity rates of a 10⁻³ dilution of Candida albicans (R1646) on 6 further rabbit corneas. This gave a mean of 49.6% in the 12 eyes studied. This dilution was taken as the CID₅₀.

Table 4 shows the infectivity rates, the mean, the standard deviation, and the standard error of a 10⁻⁴ (i.e., 4 × 10⁴ particles per ml) dilution of Candida albicans (R1646) on 6 rabbit corneas 48 hours after inoculation. This dilution scored a mean infectivity of 94.14% after 48 hours. This was accepted as approximating to CID₁₀₀. In the corneal macrophotograph (Fig. 6) taken 48 hours after inoculation with the 10⁻⁴ dilution discrete rounded lesions are visible at all sites of inoculation, without spread to involve the centre of the circles.
Development of quantitative methods of measuring antifungal drug effects in the rabbit cornea

Prophylaxis
The prophylactic effect of applying 1% clotrimazole or 1% miconazole drops to the cornea of the rabbits for 2 consecutive times hourly before inoculation with the Candida albicans (R1646) at a dilution of 4 × 10^4 particles/ml is shown in Figs. 7a and 7b.

Fig. 7a is the macrophotograph of a control eye 48 hours after inoculation, while Fig. 7b is the test eye of the same rabbit also 48 hours after inoculation. There is a marked inhibition of the development of corneal lesion in the test eye when compared with the control eye.

The infectivity rates of the test and control eyes after 48 hours of inoculation are compared in Tables 5 and 6. For the 1% clotrimazole there is a mean inhibition of establishment of lesions of 35.2%, while for 1% miconazole it is 34.76%.

Table 4  Infectivity rates, the mean, standard deviation, and standard error of a 10^4, i.e. 4 × 10^4 particles per ml, dilution of Candida albicans (R1646) on 6 rabbits' corneas 48 hours after inoculation

<table>
<thead>
<tr>
<th>Rabbit no.</th>
<th>Inoculum dilution/ml</th>
<th>% Infectivity at 48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>10^-4</td>
<td>88.28%</td>
</tr>
<tr>
<td>18</td>
<td>10^-4</td>
<td>100.0%</td>
</tr>
<tr>
<td>19</td>
<td>10^-4</td>
<td>89.07%</td>
</tr>
<tr>
<td>20</td>
<td>10^-4</td>
<td>92.19%</td>
</tr>
<tr>
<td>21</td>
<td>10^-4</td>
<td>97.66%</td>
</tr>
<tr>
<td>22</td>
<td>10^-4</td>
<td>97.66%</td>
</tr>
<tr>
<td>Mean</td>
<td>94.14</td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>4.96</td>
<td></td>
</tr>
<tr>
<td>Standard error</td>
<td>1.43</td>
<td></td>
</tr>
</tbody>
</table>

These differences between the test and control eyes are statistically significant (p < 0.005).

Fig. 8 shows the graph of the mean infectivity rates and standard errors for 6 rabbits which received prophylactic 1% clotrimazole before inoculation and their control eyes. The test eyes took on the average 4.3 days to achieve 100% corneal infectivity, while the control eyes took on the average 2.3 days to achieve 100% corneal infectivity.

The test eyes of the group of rabbits which received prophylactic treatment with 1% clotrimazole in arachis oil 48 hours after inoculation with a CID100 dilution of Candida albicans (R1646): 50% infectivity recorded.
ceived prophylactic treatment with 1% miconazole also showed a delay of 48 hours before achievement of 100% corneal infectivity. This feature has been called 'prophylactic delay'.

Clinically the test eyes looked much better even when possessing the same rate of corneal infectivity with matched control eyes; corneal oedema was much less, striation of the endothelium was less pronounced, and anterior uveitis was much less in these eyes.

THERAPY
The therapeutic effect of 1% clotrimazole when used for 10 consecutive hours daily on well established lesions (100% infectivity) is shown in Fig. 8. 1% clotrimazole cleared all the well established corneal lesions between 6 and 10 days. Notice also in that graph that when intensive treatment was established after the achievement of 100% corneal infectivity the eyes which received prophylactic treatment healed much quicker. This feature has been called 'prophylactic acceleration'. 1% miconazole showed a similar feature and when used as intensive drops for 10 consecutive hours daily on 100% infected corneal lesions produced complete resolution of the corneal lesions within 1 week.

HISTOPATHOLOGY
Mycological studies showed the growth of Candida albicans from the corneal swabs from the first day to the fourth day after inoculation with pathogens. Thereafter, when intensive treatment had been well established, the corneal and conjunctival swabs

**Table 5** The infectivity rates, means, standard deviation, and standard error of a 10⁻⁶ dilution of Candida albicans (R1646) 48 hours after inoculation in 1% clotrimazole pretreated and control cornea of 6 rabbits

<table>
<thead>
<tr>
<th>Rabbit no.</th>
<th>Inoculum dilution/ml</th>
<th>% Infectivity on eyes which received prophylaxis at 48 hours</th>
<th>% Infectivity on control eyes at 48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>10⁻⁶</td>
<td>73%</td>
<td>100%</td>
</tr>
<tr>
<td>24</td>
<td>10⁻⁶</td>
<td>36%</td>
<td>92%</td>
</tr>
<tr>
<td>25</td>
<td>10⁻⁶</td>
<td>50%</td>
<td>100%</td>
</tr>
<tr>
<td>26</td>
<td>10⁻⁶</td>
<td>70%</td>
<td>100%</td>
</tr>
<tr>
<td>27</td>
<td>10⁻⁶</td>
<td>59%</td>
<td>95%</td>
</tr>
<tr>
<td>28</td>
<td>10⁻⁶</td>
<td>88%</td>
<td>100%</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>62-67%</td>
<td>97-83%</td>
</tr>
<tr>
<td>Standard deviation</td>
<td></td>
<td>18-37</td>
<td>3-49</td>
</tr>
<tr>
<td>Standard error</td>
<td></td>
<td>7-50</td>
<td>1-42</td>
</tr>
</tbody>
</table>

**Table 6** Infectivity rates, means, standard deviations, and standard errors of a 10⁻⁶ dilution of Candida albicans (R1646) 48 hours after inoculation of 1% miconazole pretreated and control cornea of 4 rabbits

<table>
<thead>
<tr>
<th>Rabbit no.</th>
<th>Inoculum dilution/ml</th>
<th>% Infectivity on eyes which received prophylaxis at 48 hours</th>
<th>% Infectivity on control eyes at 48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>10⁻⁶</td>
<td>68-75%</td>
<td>100%</td>
</tr>
<tr>
<td>30</td>
<td>10⁻⁶</td>
<td>53-13%</td>
<td>96-88%</td>
</tr>
<tr>
<td>31</td>
<td>10⁻⁶</td>
<td>57-81%</td>
<td>100%</td>
</tr>
<tr>
<td>32</td>
<td>10⁻⁶</td>
<td>73-13%</td>
<td>100%</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>64-46%</td>
<td>99-22%</td>
</tr>
<tr>
<td>Standard deviation</td>
<td></td>
<td>11-22</td>
<td>1-56</td>
</tr>
<tr>
<td>Standard error</td>
<td></td>
<td>4-58</td>
<td>0-64</td>
</tr>
</tbody>
</table>

Fig. 8  Graph of mean infectivity rates and standard errors of control and test eyes of 6 rabbits during 15 days of prophylactic and therapeutic treatment with 1% clotrimazole in arachis oil.
Remained sterile till complete resolution of lesions. Complete resolution of lesions was confirmed by high-power slit-lamp examination of lesions, sterile mycological reports, and nonrecurrence of lesions when treatment was stopped. In one eye Streptococcus faecalis was isolated in blood agar while Candida albicans was grown in Sabouraud's medium. The Str. faecalis was interpreted as a secondary infection.

Histopathological studies confirm that the corneal lesions were due to the candidal growth and invasion of the corneal stroma. Fig. 9, which is a haematoxylin and eosin section of a 48-hour corneal lesion from Candida albicans (R1646), shows fungal invasion of the deep stroma of the cornea with vigorous inflammatory cell reaction. Fig. 10 is the same section as Fig. 9 but stained with periodic acid Schiff reagent and magnified to demonstrate some yeast cells in the corneal lesions (arrowed).

The histology of older lesions (4 days post inoculation) showed very deep invasion of the corneal stroma by the yeast cells, some lesions reaching as far as Descemet's membrane.

Fig. 9  Section of Candida albicans keratitis 48 hours after inoculation of the pathogen. (H and E, ×163).

Fig. 10  Section of Candida albicans keratitis as in Fig. 9 showing some yeast cells arrowed. (Periodic acid Schiff, ×600).
Discussion

The rabbit model of keratomycosis presented in this study is precise and easily reproducible. The large amount of data obtained from drug action on 17 micropterehne inoculation sites allow reliable statistical analysis in a small number of animals. A pathogenic strain of fungus must be chosen which regularly produces discrete lesions without confluence. This makes reading of the lesions easy. The method of scoring has been simplified to avoid complicated mathematical calculations.

The model used in this study gave a cut-off point for corneal infectivity for the Candida isolate (M1475) as 10^3 yeast particles/ml, while for the isolate (R1646) it was 10^4 yeast particles/ml. Below these levels these isolates lose their corneal infectivity. The model also demonstrates clearly the dose-response relationship of the pathogens and the host, the lower suspensions of the pathogens producing lower rates of infectivity while the higher suspensions produced higher rates of infectivity.

These observations not only demonstrate the cause and effect relationship between the pathogens and the corneal lesions, but also suggest that early identification of mycotic corneal lesions (when the population of the pathogens is low) would be the optimum period for therapeutic attack on these pathogens.

The two imidazoles studied in this paper—clotrimazole and miconazole—each had an in-vitro minimum inhibitory concentration (MIC) of 0.37 mg/l against the Candida albicans (R1646). Yet, although they both showed identical rates of lesion inhibition (prophylactic effect) of 35%, miconazole achieved complete cure of the corneal lesions within 7 days while clotrimazole took up to 10 days to resolve all corneal lesions. The superiority of miconazole over clotrimazole in the therapy of identical lesions in identical experimental conditions is thus illustrated.

This technique is highly sensitive and produces a close enough analogy with human ocular mycosis for it to be a reliable model for the study of the in-vivo effects of old and new antifungal drugs. This will be the subject of my next study.

I am grateful to the Friends of Moorfields for the fellowship grant which has enabled me to do this work. I thank Professor B. R. Jones for his advice and criticism of this work and Dr Y. M. Clayton, of the Institute of Dermatology, for making available her laboratory for this study. I also thank Elizabeth Garner and Juliana Enyiazu for secretarial help and Mr Roger Fletcher and his colleagues at the Audiovisual Department, Moorfields, for producing the photographs and diagrams. I am indebted to Mr H. Donovan, of the Institute of Ophthalmology, for computer analysis of the figures.

References