An experimental study to evaluate the effect of polymyxin E (Colistin) alone or in combination with gentamicin in McCarey-Kaufman corneal preservation medium on various drug resistant bacterial and fungal isolates

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Purpose: To assess the efficacy of the addition of polymyxin E (colistin) in the McCarey-Kaufman (MK) corneal storage solution against multi-drug resistant strains of Enterobacteriaceae, Staphylococcus aureus, and Candida spp. Methods: A standard micro broth dilution test and a checkerboard assay were performed for five multi-drug resistant (MDR) clinical strains of P. aeruginosa and five clinical strains of methicillin-resistant S. aureus (MRSA) and C. albicans against colistin and gentamicin alone and in combination. The minimum inhibitory concentration (MIC) and the fractional inhibitory concentration index (FICI) were calculated to assess the efficacy of each combination. Results: The MIC of colistin was in the range of 1–2 µg/mL for P. aeruginosa, whereas it was 256–1024 µg/mL against S. aureus. In comparison, the MIC of gentamicin was found to be 0.5–512 µg/mL and 0.5–8 µg/mL against P. aeruginosa and S. aureus, respectively. All five isolates of C. albicans did not exhibit any susceptibility to either colistin or gentamicin even at a concentration of ≥ 512 µg/mL each. The checkerboard assay was performed to evaluate the nature of the interaction of the combination of colistin and gentamicin. Based on the FICI, it was observed that the colistin and gentamicin combination has a maximum synergistic effect (FIC <0.5) in 80% (4/5) for S. aureus isolates, whereas the maximum additive effect (FIC >0.5–4) was 100% (5/5) for P. aeruginosa and the minimum additive effect was 20% (1/5) for S. aureus isolates. Antagonism (FIC ≥ 4) was not observed in any combination between the strains used in the study. Both colistin and gentamicin alone or in combination were, however, ineffective against Candida spp. Conclusion: The addition of colistin has an inhibitory effect on bacterial contamination that could be possibly caused by MDR strains and could potentially be considered as an additional additive in corneal storage media.

Key words: Colistin, endophthalmitis, MK medium, post keratoplasty infections, Pseudomonas aeruginosa

Acute endophthalmitis after keratoplasty is a devastating vision-threatening complication. The incidence of endophthalmitis after keratoplasty has been variably reported and ranges between 0.1% and 2.47% in various parts of the world.1,2 Although the donor cornea undergoes stringent evaluation before transplantation in the recipient, there exists a certain risk of infection that is often related to donor corneas harboring microorganisms.

In a previous study on acute endophthalmitis after keratoplasty over 13 years, it was observed that 50% were due to gram-negative bacilli, 7.1% due to gram-positive bacteria, 10.7% were fungal, and 32% were microbiology negative. Strikingly, the majority of the gram-negative bacteria were multi-drug resistant (MDR), being sensitive only to colistin.3,4 The most common gram-negative bacteria identified was Pseudomonas species. In contrast to this pattern, the data from the Eye Bank Association of America (EBAA) shows that fungus, especially Candida spp., is the predominant cause of infections/
the resistant microorganisms. All MDR organisms have shown good sensitivity to polymyxin E/colistin as shown from the antibiotic susceptibility tests available from the clinical samples such as corneal scrapings and vitreous samples obtained from the patients (based on clinical data). Additionally, colistin has been shown to have good activity against some fungi and Candida spp.[5] Hence, the purpose of this study was to explore the possibility of adding colistin as an adjuvant antibiotic to the MK medium and assess its efficacy against the common organisms implicated in post keratoplasty devastating infections.

**Methods**

An *in vitro* experimental study was performed. The clinical isolates of MDR *P. aeruginosa*, methicillin-resistant *S. aureus* (MRSA), and *C. albicans* were obtained from the corneal scrapings and vitreous samples of patients diagnosed with keratitis or endophthalmitis at the institute. The details are given in Tables 1–3. The isolates were cultivated and maintained in Mueller Hinton agar and potato dextrose agar, respectively. The micro broth dilution test and checkerboard assay were performed to determine the minimum inhibitory concentration (MIC) of colistin against the clinical isolates and evaluate the nature of the interaction of colistin with gentamicin that is present in the corneal storage solution-MK media.

**Cultivation of bacterial and yeast isolates**

Clinical isolates of *P. aeruginosa* (*n* = 5), *S. aureus* (*n* = 5), and *Candida albicans* (*n* = 5) were obtained. Bacterial and yeast isolates were cultivated at 37°C in Mueller Hinton agar and potato dextrose agar (HIMEDIA, India), respectively. The bacterial and the yeast suspensions were prepared by suspending 3–5 well-isolated colonies into Mueller Hinton broth and RPMI-1640 media, respectively, and adjusted to a turbidity equivalent to McFarland Standard 1–5 × 10⁸ CFU/mL and 1–5 × 10⁶ Colony forming units (CFU)/mL. The McFarland standard is further diluted to a final concentration of 5 × 10⁶ CFU/mL and 0.5–2.5 × 10⁵ CFU/mL for bacteria and yeast, respectively.

**Preparation of drugs for susceptibility testing**

The antimicrobial agents used in the study were colistin (Cipla, India) and gentamicin (Abbott, India). The standard forms of the drugs were obtained, and stock solutions of the individual drugs were prepared as a twofold concentration of

### Table 1: Clinical and demographical details of patients from which *P. aeruginosa* was isolated

| Isolates | Age and Sex | Sample    | Diagnosis             | Antibiotic susceptibility tests                        |
|----------|-------------|-----------|-----------------------|-------------------------------------------------------|
| 1        | 44-Male     | Corneal scraping | Graft infiltrate     | Penicillin - R*                                        |
|          |             |           |                       | Cephalosporins - R                                    |
|          |             |           |                       | Aminoglycosides - S†                                   |
|          |             |           |                       | Tigecycline - R                                        |
| 2        | 53-Male     | Corneal scraping | Perforated corneal ulcer | Penicillin - R                                        |
|          |             |           |                       | Cephalosporins - R                                    |
|          |             |           |                       | Aminoglycosides - S                                   |
|          |             |           |                       | Tigecycline - R                                        |
| 3        | 54-Female   | Corneal scraping | Bacterial endophthalmitis | Penicillin - R                                        |
|          |             |           |                       | Cephalosporins - R                                    |
|          |             |           |                       | Aminoglycosides - R                                   |
|          |             |           |                       | Tigecycline - R                                        |
| 4        | 36-Male     | Corneal scraping | Microbial keratitis  | Penicillin - R                                        |
|          |             |           |                       | Cephalosporins - R                                    |
|          |             |           |                       | Aminoglycosides - S                                   |
|          |             |           |                       | Tigecycline - R                                        |
| 5        | 59-Female   | Vitreous   | Endogenous endophthalmitis | Penicillin - R                                        |
|          |             |           |                       | Cephalosporins - R                                    |
|          |             |           |                       | Aminoglycosides - R                                   |
|          |             |           |                       | Tigecycline - R                                        |

*R-Resistant; †S-Susceptible

### Table 2: Clinical and demographical details of patients from which *S. aureus* was isolated

| Isolates | Age and sex | Sample    | Diagnosis               | Antibiotic susceptibility tests                        |
|----------|-------------|-----------|-------------------------|-------------------------------------------------------|
| 1        | 57-Male     | Corneal scraping | Persistent corneal defect | Cefoxitin screen – Positive                            |
|          |             |           |                         | Oxacillin - R*                                        |
| 2        | 56-Male     | Corneal scraping | Perforated corneal ulcer | Cefoxitin screen – Positive                            |
|          |             |           |                         | Oxacillin - R                                          |
| 3        | 18-Male     | Corneal scraping | Microbial keratitis     | Cefoxitin screen – Positive                            |
|          |             |           |                         | Oxacillin - R                                          |
| 4        | 37-Male     | Corneal scraping | Pythium keratitis       | Cefoxitin Screen – Positive                            |
|          |             |           |                         | Oxacillin - R                                          |
| 5        | 39-Male     | Corneal scraping | Fungal keratitis        | Cefoxitin screen – Positive                            |
|          |             |           |                         | Oxacillin - R                                          |

*R-Resistant
the final working concentration. The antibiotic solutions were then stored at 5°C until the time of use.

**Determination of MIC of colistin and gentamicin alone by micro broth dilution method**

The MIC of colistin and gentamicin against the bacterial and yeast cultures was determined by micro broth dilution method as outlined by Clinical and Laboratory Standards Institute (CLSI) guidelines, 2012. Briefly, the drugs were serially diluted in Mueller Hinton broth to obtain their final working concentrations. The concentration of colistin ranging from 0.25–16 μg/mL, 8–1024 μg/mL, and 2–512 μg/mL was used against *P. aeruginosa*, *S. aureus*, and *C. albicans*, respectively. The concentration of gentamicin ranging from 0.5–512 μg/mL was used against *P. aeruginosa* and *C. albicans*, and 0.25–16 μg/mL was used against *S. aureus*. The micro broth dilution method was performed on a 96-well plate. A 100 µL of antimicrobial agent of varying concentrations was added to the plate along with 100 µL of the prepared bacterial or yeast inoculum having a density of 5 × 10⁵ CFU/mL and 0.5–2.5 × 10⁵ CFU/mL, respectively. Sterility and a growth control were added to the 96-well plates to validate the experiment. The plate was then incubated at 37°C for 24 h. The MIC, which is the lowest concentration of the drug that completely inhibits the growth of the organism, was determined for each drug individually against their respective organism. The quality control strains used were, ATCC 27853, ATCC 29213 for *P. aeruginosa* and *S. aureus*, respectively, to monitor the test performance.

**Determination of drug interaction by checkerboard assay**

Based on the results obtained in the micro broth dilution test, a checkerboard assay was performed to assess the nature of the interaction between the drugs. Stock solutions of individual drugs were prepared as a fourfold concentration of the final working concentration. The concentration of colistin and gentamicin ranging from 0.0625–8 μg/mL to 0.0625–32 μg/mL, respectively, was used against *P. aeruginosa* and *S. aureus*. These concentration ranges were established based on the MIC values of the individual drugs against their respective organisms. As shown in Fig. 1, one of the drugs was dispensed along the abscissa and the other along the ordinate in such a way that each well contained a combination of both drugs to get their final concentration. The bacterial inoculum diluted to a final concentration of 5 × 10⁵ was added to each well containing a combination of drugs. Sterility and growth control were also added to the 96-well plates and incubated at 37°C for 24 h. The fractional inhibitory concentration (FIC) of the drug combination was calculated against each organism.

The equation \( \Sigma \text{FIC} = \text{FIC}_A + \text{FIC}_B = A/\text{MIC}_A + B/\text{MIC}_B \) was used to calculate the FIC, where A is the MIC of antibiotic A in combination, MIC of the MIC of antibiotic A alone and B is the MIC of antibiotic B in combination, MIC of antibiotic B alone. A combined FIC value of ≤0.5 indicates synergy, a value of >0.5–4 indicates additive effect/indifference, and a value of >4 was suggested to be antagonism. [9]

**Evaluation of endothelial toxicity by cytotoxic bioassay**

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was performed to examine the toxicity of colistin on endothelial cells. A 96-well plate was seeded with 10⁵ cells/well. The cells were treated with different concentrations of colistin diluted in the MK media ranging from 1 to 32 μg/mL and incubated for 24 h. The cells were then treated with MTT solution and incubated for 1 h. During this period, the MTT, a yellow tetrazolium salt gets reduced by succinate dehydrogenase in the mitochondria of the viable cells. DMSO is then added to the cells to solubilize the MTT crystals that on reduction form purple formazan crystals, indicating the proportion of viable cells. The absorbance of each well was recorded at a wavelength of 575 nm using a spectrophotometer. The cell cytotoxicity was calculated using the following formula:

\[ \text{Cell viability percentage} = \frac{\text{Mean absorbance of test well}}{\text{Mean absorbance of control well}} \times 100. \]

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**Table 3: Clinical and demographical details of patients from which *C. albicans* was isolated**

| Isolates | Age and sex | Sample     | Diagnosis             | Antibiotic susceptibility tests |
|----------|-------------|------------|-----------------------|--------------------------------|
| 1        | 36-Male     | Corneal scraping | Microbial keratitis  | Flucytosine - S† |
|          |             |            |                       | Echinocandins - S |
|          |             |            |                       | Amphotericin B - S |
|          |             |            |                       | Gentamicin - S |
| 2        | 65-Female   | Vitreous   | Chronic endophthalmitis | Flucytosine - S |
|          |             |            |                       | Echinocandins - S |
|          |             |            |                       | Amphotericin B - S |
| 3        | 49-Female   | Corneal scraping | Blunt trauma        | Flucytosine - S |
|          |             |            |                       | Echinocandins - S |
|          |             |            |                       | Amphotericin B - S |
| 4        | 4-Male      | Corneal scraping | Fungal keratitis   | Flucytosine - S |
|          |             |            |                       | Echinocandins - S |
|          |             |            |                       | Amphotericin B - S |
| 5        | 73-Female   | Corneal scraping | Microbial keratitis | Flucytosine – S |

*S-Susceptible
Statistical analysis was performed following the experiment using one-way analysis of variance (ANOVA) following post hoc Tukey’s test using Prism 5 software. Any P value ≤0.05 was considered statistically significant.

Results

The MIC of drugs alone and in combination are shown in Tables 4 and 5. It was observed that the MICs of colistin ranged from 1–2 µg/mL to 256–1024 µg/mL against P. aeruginosa (as shown in Fig. 2) and S. aureus, respectively, and the MICs of gentamicin ranged from 0.5–512 µg/mL to 0.5–8 µg/mL against P. aeruginosa and S. aureus, respectively. The fractional inhibitory concentrations of the combination of colistin and gentamicin against P. aeruginosa and S. aureus are shown in Tables 4 and 5, suggesting that the combination of colistin and gentamicin has an additive effect in 100% (5/5) of the P. aeruginosa and 20% (1/5) of the S. aureus and a synergistic effect in 80% (4/5) of the S. aureus isolates, as shown in Fig. 3 and Table 6. No antagonism was observed in any case. The MICs of colistin and gentamicin individually were determined for C. albicans and the MICs were observed to be >512 µg/mL each, suggesting that C. albicans is not susceptible to colistin and gentamicin either individually or in combination. Based on the MIC obtained in the micro broth dilution test, a cytotoxic test (MTT assay) was performed to evaluate the safety of colistin on the corneal endothelial cells. As shown in Fig. 4 and Table 7, at any given concentration ranging from 32 µg/mL to 1 µg/mL, there was no toxic effect observed on corneal endothelial cells, suggesting that colistin does not have any significant toxic effect on corneal endothelial cells (P ≠ 0.05).

Discussion

The study aimed to assess the efficacy of colistin in the MK medium against three types of microorganisms, MDR P. aeruginosa, MRSA, and Candida spp. Colistin is a polycationic peptide antimicrobial produced by the Bacillus polymyxa subspecies colistinus. The cationic polypeptide of colistin acts

**Table 4**: Indicates the MIC and FICI of colistin and gentamicin alone and in combination against each isolate of P. aeruginosa

| Strain No. | MIC of colistin by BMD (µg/mL) | MIC of colistin by VITEK (µg/mL) | MIC of gentamicin by BMD (µg/mL) | MIC of gentamicin by VITEK (µg/mL) | ΣFICI of colistin + gentamicin |
|-----------|-------------------------------|----------------------------------|----------------------------------|-----------------------------------|-------------------------------|
| L-1616    | 1                             | ≤0.5                             | 0.5                              | ≤1                                | 1.97                          |
| L-1761    | 2                             | ≤0.5                             | 0.5                              | ≤1                                | 1.70                          |
| L-2436    | 2                             | ≤0.5                             | 512                              | ≥16                               | 0.96                          |
| L-2467    | 1                             | ≤0.5                             | 2                                | 2                                 | 0.98                          |
| L-2719    | 2                             | 2                                | 256                              | ≥16                               | 1.22                          |

*BMD- Broth micro dilution. *MIC- Minimal inhibitory concentration. **FICI- Fractional inhibitory concentration index

**Table 5**: Indicates the MIC and FICI of colistin and gentamicin alone and in combination against each isolate of S. aureus

| Strain No. | MIC of colistin by BMD (µg/mL) | MIC of gentamicin by BMD (µg/mL) | MIC of gentamicin by VITEK (µg/mL) | ΣFICI of colistin + gentamicin |
|-----------|-------------------------------|----------------------------------|-----------------------------------|-------------------------------|
| L-1416    | 1024                          | 1                                | ≤0.5                              | 0.50                          |
| L-1737    | 256                           | 8                                | ≥16                               | 0.11                          |
| L-1791    | 256                           | 0.5                              | ≤0.5                              | 2                             |
| L-1792    | 256                           | 4                                | 8                                 | 0.24                          |
| L-2103    | 512                           | 2                                | ≤0.5                              | 0.25                          |

*BMD- Broth micro dilution. *MIC- Minimal inhibitory concentration. **FICI- Fractional inhibitory concentration index
Table 6: The percentage of synergism and additive effects in *P. aeruginosa* and *S. aureus* against the combination of colistin and gentamicin

| Isolate       | % Synergism | % Additive | % Antagonism |
|---------------|-------------|------------|--------------|
| *P. aeruginosa* (n=5) | 0 (0)       | 100 (5)    | 0 (0)        |
| *S. aureus* (n=5)    | 80 (4)      | 20 (1)     | 0 (0)        |

on the lipopolysaccharide of the outer membrane of the gram-negative bacteria, displaces their Ca\(^{2+}\) and Mg\(^{2+}\) ions that maintain the stability of the cell membrane, causes leakage of cell contents, and increases the permeability of the cell membrane, and finally leads to the death of the bacteria. Apart from its activity against gram-negative bacteria, colistin has been shown to have an inhibitory influence on gram-positive bacteria such as *S. aureus*.\(^{[5]}\) In a study conducted on the activity of colistin on gram-positive bacteria, *Paenibacillus polymyxa*,\(^{[6]}\) it was observed that the concentration of colistin required to inhibit the growth of gram-positive bacteria is a hundred times greater than that to inhibit gram-negative bacteria. It was found that higher concentrations of colistin tend to act on the negatively charged teichoic acids present on the thick peptidoglycan layer of gram-positive bacteria and disrupt the bacterial cell wall leading to cell death. A similar mechanism can be possible in the case of other gram-positive bacteria such as *S. aureus*.\(^{[7]}\) Colistin was also observed to have anti-fungal activity against *C. albicans*.\(^{[5]}\)

Although the risk of donor cornea contamination has reduced significantly with the aseptic protocols and practices adopted during cornea retrieval, micro-organisms can survive and escape the detection by clinical evaluation methods and thus, have a potential to cause post keratoplasty infections. Further, the growing resistance of gram-negative organisms to gentamicin, the antibiotic in the cornea, is a cause of concern. This can increase the load of resistant microbes in donor corneas retrieved from hospital-based cornea retrieval programs as the antibiotic in the corneal preservation medium (gentamicin), will be ineffective in this situation. Hence, we wanted to test
the efficacy of an additional anti-microbial agent, colistin, which has activity against MDR organisms, especially against Enterobacteriaceae, which have been the most common cause of post keratoplasty endophthalmitis.

The present study has demonstrated that the MICs of colistin were in a range of 1–2 µg/mL against *P. aeruginosa*, 256–1024 µg/mL against *S. aureus*, and >512 µg/mL against *C. albicans*. We have also evaluated the nature of the interaction of colistin with gentamicin because it is the only antibiotic present in the MK media. Our data on in vitro combinational assay suggests that colistin and gentamicin have an additive effect on 100% of the tested isolates of *P. aeruginosa* and synergistic and additive effects on 80% and 20% of *S. aureus*, respectively. Colistin, however, did not show activity either individually or in combination with gentamicin against *C. albicans* at the concentration tested.

A study by Hindler et al., showed the MIC of colistin against *P. aeruginosa* in accordance with the current study, validating the reproducibility of our experiment. Also, a combination of colistin and gentamicin has shown to have a synergistic effect on *P. aeruginosa* in a study conducted by Rynn et al. In our study, the MIC exhibited by colistin against *C. albicans* was observed to be > 512 µg/mL, whereas a slightly lower MIC was described by Schwarz et al. This could possibly be due to the higher resistance exhibited by the strains used in our study. Further, colistin was ineffective against *C. albicans* even when used in combination with gentamicin. This suggests that colistin cannot interact with gentamicin but possibly could be used in combination with an antifungal agent to produce a fungicidal effect against *C. albicans* as was shown in a study by Schwarz et al.

The addition of any antibiotic to the corneal preservation medium should be tested for its effect on the corneal endothelium. The in vitro cytotoxicity tests at the concentration of colistin employed in the current study did not seem to have a toxic effect on the endothelium.

The study demonstrates the in vitro efficacy of colistin as an additional antibiotic to gentamicin. However, the results of this laboratory-based study should be investigated further to evaluate the safety and efficacy in donor corneas inoculated with micro-organisms.

### Financial support and sponsorship
Nil.

### Conflicts of interest
There are no conflicts of interest.

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### Table 7: Depicts the percentage of endothelial cell viability for a range of colistin concentrations added to MK media

| Conditions                        | OD AT 575 nm | Average   | Standard deviation | Cell viability |
|----------------------------------|-------------|-----------|--------------------|----------------|
| Cells only (Opti-MEM) - control 1| 0.335       | 0.358     | 0.3465             | 100            |
| Cells only (MK Media) - control 2| 0.386       | 0.447     | 0.4165             | 120.2020202    |
| 32 µg/mL colistin + MK media     | 0.361       | 0.409     | 0.385              | 111.111111     |
| 16 µg/mL colistin + MK media     | 0.438       | 0.433     | 0.4355             | 125.6854257    |
| 8 µg/mL colistin + MK media      | 0.474       | 0.402     | 0.438              | 126.4069264    |
| 4 µg/mL colistin + MK media      | 0.428       | 0.431     | 0.4295             | 123.935824     |
| 2 µg/mL colistin + MK media      | 0.472       | 0.374     | 0.423              | 122.079221     |
| 1 µg/mL colistin + MK media      | 0.435       | 0.412     | 0.412              | 122.222222     |