

1 **Antimicrobial peptides in human corneal tissue of fungal keratitis patients**

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39 **Precis**

40 HBD-1 and -2 were consistently expressed in fungal keratitis samples. While HBD-3, -9, S100A7,
41 and LL-37 showed variable expression pattern. The results indicate the therapeutic potential of
42 recombinant or linear AMPs against fungal pathogens.

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64 **ABSTRACT**

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66 **Background:** Fungal keratitis (FK) is the leading cause of unilateral blindness in the developing
67 world. Antimicrobial peptides (AMPs) have been shown to play an important role on human ocular
68 surface (OS) during bacterial, viral, and protozoan infections. Here, our aim was to profile a spectrum
69 of AMPs in corneal tissue from FK patients with active infection and after healing.

70

71 **Methods:** OS samples were collected from patients at presentation by impression cytology and
72 scraping. Corneal button specimens were collected from patients undergoing therapeutic penetrating
73 keratoplasty for management of severe FK or healed keratitis. Gene expression of human beta-
74 defensin (HBD)-1, -2, -3 and -9, S100A7, and LL-37 was determined by quantitative real-time
75 polymerase chain reaction (qPCR).

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77 **Results:** Messenger RNA expression (mRNA) for all AMPs was shown to be significantly
78 upregulated in FK samples. Levels of HBD-1 and -2 mRNA were found to be elevated in 18/20 FK
79 samples. Whereas mRNA for HBD-3 and S100A7 was upregulated in 11/20 and HBD9 was increased
80 in 15/20 FK samples. LL-37 mRNA showed moderate upregulation in 7/20 FK samples compared to
81 controls. In healed scar samples, mRNA of all AMPs was found to be low and matching the levels in
82 controls.

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84 **Conclusion:** AMP expression is a consistent feature of FK, but not all AMPs are equally expressed.
85 HBD-1 and -2 are most consistently expressed and LL-37 the least, suggesting some specificity of
86 AMP expression related to FK. [This information will be useful in developing strategies](#)
87 [The outcomes will form the basis to use HBDs sequence as a template for designing FK-specific peptide](#)
88 [therapeutics for use of AMPs in treating FK.](#)

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INTRODUCTION

Fungal keratitis (FK) is the commonest cause of corneal blindness in developing world. The prevalence of FK has been linked to geographical climate¹. In India, it was estimated that up to 35% of patients that present with infective keratitis were culture positive for mycotic organisms^{2,3}. The principal risk factor for FK in more than 70% of these cases was reported to be vegetative ocular injury³. Increasing reports of FK cases from countries with temperate conditions have added to the infection-related incidence of blindness globally^{4,6}. In developed countries, incidence of FK cases is also increasing and mainly associated with contact-lens use and ocular trauma⁷. In the United Kingdom, FK incidence increased from 4.5 cases per year (between 1994 to 2006) to 14 cases per year (between 2007 to 2014)⁵. In the midwestern region of the United States, 16% cases of infective keratitis (between 1999 to 2013) were identified as FK with poor healing and major complications⁸. The socio-economic impact of this disease has been significantly high because it predominantly affects individuals in working age group⁹⁻¹¹. Clinical features of FK often overlap with bacterial keratitis and frequently these are difficult to diagnose¹². Poor penetration and lack of effective anti-fungal agents has further compounded the problem of FK¹³⁻¹⁵. Therefore, to seek alternative therapies, current research in this field has been mainly focused on understanding of mechanisms by which host immunity responds to fungi and yeast.

Antimicrobial peptides (AMPs) are naturally occurring host defence proteins with broad-spectrum antimicrobial activity against bacteria, fungi and viruses¹⁶⁻¹⁸. They play an important role in innate immunity and are known to be expressed at epithelial surfaces of the human body. We were the first group to provide evidence for the presence of AMPs at the ocular surface¹⁹ and profiled the range of AMPs (human beta-defensin (HBD)-1 to 3, HBD-9 and cathelicidin (LL-37)) at the human ocular surface and their expression in corneal infections²⁰⁻²². We also elucidated the signaling mechanisms involved in RNase-7 and HBD-9 secretion from human corneal epithelial cells^{23,24}.

119 Antifungal activity of AMPs (such as defensins and cathelicidin) is well known²⁵⁻²⁷ through animal
120 studies, but the profile of human AMPs at the ocular surface in response to fungal infections has not
121 been elucidated. In this study, we profiled the gene expression of well-characterised human AMPs in
122 corneal specimens during active phase of infection and after healing.

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125 **MATERIALS AND METHODS**

126 Research was conducted in accordance with the tenets of the Declaration of Helsinki. Study was
127 approved by the Institutional Review Board of Hyderabad Eye Research Foundation (**Ethics code:**
128 **2016-13-CD-13**), L.V. Prasad Eye Institute (LVPEI), Bhubaneswar, India. Informed consent was
129 obtained from all patients prior to collection of samples, which included scrapes/ impression cytology
130 from patients with active fungal keratitis, corneal buttons (part of) from patients that were subjected
131 to therapeutic penetrating keratoplasty (tPK) for fungal keratitis not responding to medical
132 management and corneal tissue from patients with non-inflammatory corneal scar that underwent
133 optical penetrating keratoplasty (controls).

134

135 **Study design:**

136 A prospective consecutive case series of patients with fungal keratitis

137 Inclusion criteria

- 138 • Patients diagnosed clinically as suffering from FK and confirmed by standard microbiology
139 and/or histopathology techniques.
- 140 • Patients with active FK who had received no treatment or were unresponsive to treatment.
- 141 • Patients of 18 years of age or older.
- 142 • Patients able to give informed consent.

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144 Exclusion criteria

- 145 • Patients with mixed fungal and bacterial keratitis

- 146 • Patients on topical or systemic steroid treatment
147 • Patients on immunosuppressive treatment or known to be immunosuppressed
148 • Patients presenting with non-infectious causes of ocular inflammation
149 • Patients with viral keratitis

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151 **Sample collection:**

152 Corneal scrapes were collected using standard techniques with sterile Bard Parker blade number 15²⁸.
153 Impression cytology (IC) was performed to collect superficial layers of corneal epithelium using
154 cellulose acetate ester discs, as previously reported²⁰. Part of corneal button (CB) from patients
155 undergoing therapeutic or optical PK was collected in 600 µL RNeasy lysis solution (Qiagen, Germany)
156 on ice and then transferred to RNeasy lysis buffer prior to storage at -80°C.

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158 **Total RNA isolation and Reverse transcription:**

159 Corneal tissue in RNeasy lysis buffer was homogenized using a tissue raptor (Qiagen, Germany) for 60
160 seconds on ice. Total RNA was isolated from CB, IC samples, and corneal scrapes using RNeasy
161 Mini Kit (catalog No. 74104; Qiagen, Germany) according to manufacturers' instructions, including
162 the optional DNase step. Isolated RNA was quantitated using Biospectrophotometer (Eppendorf,
163 Germany). 200ng total RNA was reverse transcribed to complementary deoxyribonucleic acid
164 (cDNA) using Eurogentec Reverse Transcription Core Kit (RT-RTCK-03, Eurogentec, Belgium) as
165 per manufacturers' instructions. No RT-enzyme control samples were also prepared.

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167 **Quantitative real-time polymerase chain reaction (qPCR):**

168 Quantitative PCR was conducted for selected AMPs (Table 1) using Taqman probe chemistry.
169 Taqman assays specific to AMPs, hypoxanthine-guanine phosphoribosyltransferase [HPRT] and
170 appropriate controls were run in duplicate in a 96-well plate in the Mx3005p real-time PCR
171 instrument (Agilent technologies, Milton Keynes, UK). Briefly, template cDNA was initially diluted
172 to 1 in 5 using nuclease-free water. A 20 µL reaction mix was prepared for each well as per

173 instructions for Taqman Gene Expression mastermix (Applied Biosystems, Waltham, MA). Each
174 reaction mixture comprised of 10 μ L of 2x mastermix, 1 μ L of 20x taqman assay, 5 μ L of diluted
175 cDNA and 4 μ L of nuclease-free water. All probes used in this study were template specific. However,
176 to rule out any genomic amplification, appropriate no-RT controls were also included against each
177 gene probe. C_T values were acquired using MxPro Software version 5.0 and further analysed by delta-
178 delta C_T comparative method²⁹.

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180 **Statistical analysis:**

181 The qPCR data was subjected to statistical analysis using Prism version 8.1 software (GraphPad
182 software, San Diego, CA) with alpha set at $p \leq 0.05$. Due to differences in the group size, we
183 performed unpaired Welch's unequal variances *t*-test for control versus active and active versus
184 healed groups.

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186

187 **RESULTS**

188 As shown in table 2, a total 26 samples were collected. These included a part of CB (n=17), scrapings
189 (n=5) and IC (n=4). Of CB specimens, 3 were grouped as 'controls' (patients' that required optical
190 PK for management of non-inflammatory corneal scar); 11 were grouped as 'active FK' (patients'
191 with severe FK that required tPK) and 3 were grouped as 'healed' keratitis (patients' that required PK
192 post-medical management for FK). Table 2 mentions the day of collection of corneal button samples
193 (Column no.8). CB tissue of active FK was collected between day 1 to 30 from the day of
194 presentation and those from the healed group between 6-12 months from the day of presentation.
195 Scraping and IC samples (5+4) were also included under the group of 'active FK' and were collected
196 between day 0 to 3 from the day of presentation, before commencement of antifungal therapy. In
197 total, we studied n=3 in controls; n=20 in active FK and n=3 in healed group. As shown in table 2,
198 final diagnosis was based on positive growth of fungi in cultures from corneal scrapings and/or on
199 histopathology evaluation. Of n=23 cases of fungal keratitis, 3 samples showed no growth on culture,

200 however, they were later confirmed as fungal by aetiology on histological evaluation. In addition,
201 2/23 patients presented with a perforation or an impending perforation were not scraped but later
202 confirmed as FK, on histopathology. A range of fungi were identified but fusarium species was the
203 most common (Table 2).

204

205 **Variable expression of AMPs in active FK and healed samples**

206 HBD-1, -2, and -9 and LL-37 were shown to be constitutively expressed in all control samples,
207 whereas mRNAs for HBD-3 and S100A7 were expressed at a very low level in controls. As shown in
208 figure 1, all AMPs were significantly increased during active FK. Notably, in healed samples, their
209 mRNA expression was found to be at a similar level to those in controls. Levels of mRNA expression
210 is denoted as mean \pm standard deviation.

211

212 HBD-1 and -2 mRNA were significantly increased in 18/20 active FK samples. HBD-1 was
213 upregulated by 12.03 ± 9.06 fold ($p < 0.0001$) and HBD-2 was elevated by 254.7 ± 335.82 fold
214 ($p = 0.003$) in FK compared to controls. However, in healed samples, these levels were significantly
215 reduced with HBD-1 at 2.19 ± 1.72 fold ($p = 0.0003$) and HBD-2 at 0.23 ± 0.28 fold ($p = 0.003$)
216 compared to active FK.

217

218 The level of HBD-3 and S100A7 mRNAs were elevated in 11/20 FK samples, whereas HBD9 mRNA
219 was upregulated (> 2 -fold) in 15/20 FK samples. HBD-3 was increased by 5.54 ± 8.28 fold ($p = 0.007$)
220 and HBD-9 was elevated by 31.83 ± 51.15 fold ($p = 0.018$) in FK. Similar to other defensins, HBD-3
221 and HBD-9 also showed baseline expression in healed samples with levels at 0.01 ± 0.02 fold
222 ($p = 0.007$) and 0.51 ± 0.40 fold ($p = 0.013$), respectively. S100A7 mRNA expression was shown to be
223 increased by 61.89 ± 95.73 fold ($p = 0.009$) in FK samples compared to controls. Whereas in healed
224 samples, mRNA levels for S100A7 were reduced to 0.19 ± 0.34 fold ($p = 0.009$) compared to FK.

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226 Of all AMPs, LL-37 mRNA was moderately increased in FK showing elevated expression ($4.05 \pm$
227 7.41 fold; $p=0.047$) only in 7/20 samples. In healed samples, LL-37 was expressed at low levels (1.16
228 ± 1.01 fold; *not significant*) matching the mRNA levels in controls.

229 **DISCUSSION**

230 AMPs have attracted special attention as potential therapy for microbial infections due to their unique
231 mode of action compared to available antimicrobial therapies³⁰. In the last two decades, we and others
232 have extensively demonstrated an essential role of human AMPs in microbial keratitis^{17 19-23 31-33}. In
233 corneal epithelial cells, expression of HBD-2, HBD-3 and LL-37 were shown to be significantly
234 elevated in response to infection with *Fusarium solani*³⁴ and *Candida albicans*³⁵, respectively.
235 Elevated levels of cytokines and other innate immune receptors was previously demonstrated in
236 human corneal specimen from patients with *Aspergillus flavus* and *Fusarium solani* infection³⁶.
237 Moreover, increased susceptibility to corneal infections by *Aspergillus fumigatus*, *Fusarium solani*
238 and *Candida albicans* was previously demonstrated in mice deficient in cathelin-related antimicrobial
239 peptide (CRAMP) and murine β -defensins (mBD-1 to -4)^{37 38}.

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241 In this study, we demonstrated an increased pattern of AMPs (HBD-1 to -3, HBD-9, S100A7 and LL-
242 37) expression in corneal specimen during active infection. Notably, in healed specimen, mRNA of
243 all AMPs was found to be at a basal level. A similar phenomenon has been noted in our previous
244 demonstration of HBD-3 and HBD-9 mRNA levels in OS specimen collected during active bacterial
245 keratitis and following complete healing³¹. Moreover, in an animal model of *Candida albicans*
246 keratitis, CRAMP and β -defensins (mBD-1 and -2) have demonstrated a variable expression at the
247 onset of disease but returned to their normal level upon healing at day 7 post-infection³⁸. In *Fusarium*
248 *solani* keratitis mouse model, mBD-3, mBD-4, mBD-14 and CRAMP were shown to be significantly
249 increased by day 3 post-infection, which then started to decrease with reduction in disease severity
250 reaching to baseline upon healing³⁷. From above studies, it could be inferred that rapid normalisation
251 of AMPs expression following healing occurs and is likely to be of importance considering the fact
252 that high levels of AMPs could elicit toxic responses on ocular surface.

253

254 Previously we have demonstrated a significant downregulation of HBD9 expression in specimen from
255 patients with bacterial keratitis, acanthamoeba keratitis, viral keratitis and dry-eye disease²⁰.

256 Interestingly, in current study, we have noted an elevated pattern of HBD9 expression in active FK
257 samples. This unique response of HBD9 during fungal infection suggest a potential anti-fungal
258 function of this AMP. We have generated the recombinant protein of HBD9 using *E. coli* expression
259 system and demonstrated that both recombinant HBD9 protein and its full-length synthetic linear
260 peptide were unstable, which was attributed to its proline rich C-terminus. Failure to keep the protein
261 or its linear peptide in solution has thus far prevented us to test the antimicrobial efficacy of HBD9
262 against disease-causing pathogens (unpublished observations).

263

264 S100A7 was first isolated from skin of Psoriasis patients hence it is also known as Psoriasin³⁹.

265 S100A7 has been shown to express constitutively in different regions of anterior segment of the eye⁴⁰.

266 Interestingly, cysteine-reduced form of S100A7 protein has been previously shown to exhibit potent
267 antifungal activity against dermatophytes and filamentous fungi⁴¹. Whereas both native and reduced
268 forms of S100A7 showed activity against the yeast, *C. albicans*⁴¹. A previous study has demonstrated
269 the increased levels of S100A7 in vaginal biopsies from patients with *C. albicans* vulvovaginitis⁴².

270 Similar increased levels of S100A7 were also noted in this study. Further research using gene
271 knockout experiments would highlight the importance of S100A7 in host defense to fungal infections
272 at the ocular surface.

273

274 Although FK is slowly progressive compared to bacterial keratitis, the rate of corneal perforation has
275 been reported to be high in FK cases⁴³. AMPs are potent chemo attractants and capable of eliciting
276 adaptive immunity³⁰. Thus, in addition to direct killing of microbes, increased level of AMPs during
277 FK could potentially increase neutrophil infiltration, which might contribute towards tissue damage,
278 melting, and scarring. A recent report has demonstrated that deficiency of CRAMP and mBD-3 and -4
279 increased the susceptibility to *F. solani* keratitis and led to excessive infiltration of neutrophils which
280 was attributed to high levels of KC (a neutrophil chemokine) in corneal tissue³⁷. Similarly, in *C.*

281 *albicans* keratitis model, deletion of CRAMP was also shown to increase yeast burden, neutrophil
282 recruitment and levels of IL-1 β and MIP-2⁴⁴. In stark contrast to keratitis model, severity of *C.*
283 *albicans* infection in oral mucosa in mBD-1 knockout mice have been associated with low neutrophil
284 recruitment and reduced levels of IL-1 β , KC, IL-17A and IL-17F⁴⁵. In mice cornea, the specific
285 function of neutrophil derived calprotectin (S100A8/A9) in clearance of *A. fumigatus* has been
286 demonstrated. Unlike cationic AMPs, calprotectin was shown to exhibit antifungal activity *via*
287 chelation of zinc and manganese which retards *A. fumigatus* growth⁴⁶.

288

289 Our results corroborate previous studies on AMP regulation during fungal infection in animals. This
290 further implicates a pivotal role for AMPs in OS defense against fungal pathogens. However, their
291 diverse function in modulation of neutrophil infiltration and inflammation in cornea and other
292 mucosal sites during infection still remains unclear. Though this is the first study on AMPs in human
293 FK further studies are highly warranted to understand the mechanisms of immune activation of AMPs
294 during FK. The limitation of this study was that it only involved assessment of gene expression of
295 AMPs in active and healed groups. A follow-up study addressing the cellular source of these AMPs in
296 active FK samples will further enhance the understanding of function of key AMPs towards
297 fungi/yeast.

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465 **Figure legend**

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467 **Figure 1.** Gene expression of antimicrobial peptides in corneal tissue from patients with fungal
468 keratitis. Relative fold change of A) HBD1, B) HBD2, C) HBD3, D) HBD9, E) S100A7 and F) LL-
469 37 in control, active FK and healed groups. Data points represent individual patients in each group.
470 Student's t-test with Welch's correction was performed to compare control vs active FK and active
471 FK vs healed with $p \leq 0.05$ denotes statistical significance.

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474 **Tables**

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477 **Table 1.** List of TaqMan probes

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<i>Gene name</i>	<i>Assay ID</i>
<i>DEFB1 (HBD-1)</i>	Hs00608345_m1
<i>DEFB4 (HBD-2)</i>	Hs00823638_m1
<i>DEFB103(HBD-3)</i>	Hs00218678_m1
<i>DEFB109(HBD-9)</i>	Hs002760065_g1
<i>S100A7</i>	Hs00961622_m1
<i>CAMP (LL-37)</i>	Hs00189038_m1
<i>HPRT1</i>	4325801

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Table 2. Clinical diagnosis and laboratory results

No.	Age /Sex	Clinical diagnosis	Size of inf.(mm)	Scr-C/S	Sample used	Outcome / Keratoplasty	Pres to Ker	Histopathology findings	CB-C/S
1.	36 /M	Corneal scar	NA	NA	CB	NA/Y	11m	Vascularised corneal scar	NA
2.	38 /F	Corneal scar, post trauma	NA	NA	CB	NA/Y	5 yrs	Non inflammatory corneal scar	NA
3.	19 /M	Corneal scar since childhood	NA	NA	CB	NA/Y	6m	Non inflammatory corneal scar	NA
4.	86 /M	Microbial Keratitis	3.5x2.5	unid. hyaline fungus	Scrape	Resolved keratitis /ND	NA	NA	NA
5.	86 /M	Microbial Keratitis	3.5x2.5	unid. hyaline fungus	Imp	Resolved keratitis/ ND	NA	NA	NA
6.	30 /F	Corneal ulcer	TCI	<i>Aspergillus spp.</i>	Scrape	Failed to resolve/ Y	30d	Fungal corneal ulcer	NG
7.	30 /F	Corneal ulcer	TCI	<i>Aspergillus spp.</i>	Imp	Failed to resolve/ Y	30d	Fungal corneal ulcer	NG
8.	22 /F	Corneal ulcer	2x1.5	NG	Scrape	Partially resolved /Y	30d	Fungal keratitis with yeast like spores	NG
9.	36 /M	Fungal Keratitis	4x4.5	<i>Fusarium spp.</i>	Scrape	Resolved keratitis /ND	NA	NA	NA
10.	69 /M	Perforated Corneal ulcer with hypopyon	TCI	<i>Lasiodiplodia spp.</i>	Scrape	Failed to resolve/ Y	18d	Fungal corneal ulcer	NG
11.	27 /F	Microbial Keratitis	3.5x2	<i>Fusarium spp.</i>	Imp	LFU	NA	NA	NA
12.	31 /M	Corneal ulcer with hypopyon	TCI	<i>Aspergillus spp.</i>	Imp	Resolved keratitis/ Y	6m	Corneal scar	NG
13.	75 /M	Fungal Keratitis	5x2	<i>Acremonium spp.</i>	CB	Failed to resolve/ Y	30d	Fungal Corneal ulcer	NG
14.	39 /M	Microbial Keratitis	5.5	<i>Fusarium spp.</i>	CB	Failed to resolve/ Y	28d	Fungal Corneal ulcer	Y
15.	44 /M	Perforated corneal	TCI	ND	CB	Perforated corneal	0d	Fungal Corneal ulcer	Y

		ulcer				ulcer/Y			
16.	59 /M	Fungal Keratitis	TCI	<i>Fusarium spp.</i>	CB	Large corneal ulcer/Y	1d	Fungal Corneal ulcer	Y
17.	48 /M	Microbial Keratitis	3x4	<i>Aspergillus spp.</i>	CB	Large corneal ulcer/Y	6d	Fungal Corneal ulcer	NG
18.	46 /M	Microbial Keratitis	3x2	NG	CB	Large corneal ulcer/Y	4d	Fungal Corneal ulcer	Y
19.	54 /M	Fungal Keratitis	6x5	<i>Aspergillus spp.</i>	CB	Large corneal ulcer/Y	4d	Fungal Corneal ulcer	Y
20.	58 /F	Microbial Keratitis	3.5x3	<i>Unid. Dematiace ous Fungus</i>	CB	Impendin g perforati on / Y	2d	Fungal Corneal ulcer	Y
21.	56 /M	Fungal Keratitis	10x8	<i>Fusarium spp.</i>	CB	Near total infiltrate/ Y	9d	Fungal Corneal ulcer	NG
22.	44 /F	Microbial Keratitis	7x6	NG	CB	No response/ Y	5d	Fungal Corneal ulcer	NG
23.	44 /M	Perforated corneal ulcer	TCI	ND	CB	Perforate d corneal ulcer/Y	0d	Fungal Corneal ulcer	Y
24.	37 /F	Fungal Keratitis	3.5x2.5	<i>Fusarium spp.</i>	CB	Resolved keratitis/ Y	7m	Corneal scar	NG
25.	52 /M	Fungal Keratitis	TCI	<i>Burkholder ia spp.</i>	CB	Resolved keratitis/ Y	10m	Corneal scar	NG
26.	26 /M	Microbial keratitis	8x5	<i>pseudo allescheria boydii</i>	CB	Resolved keratitis/ Y	10m	Corneal scar	NG

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Abbreviations: Inf- infiltrate; M/F- male/female; CB-corneal button; Y-yes; Pres to Ker- duration between the date of first presentation and date of keratoplasty; d/m/yrs-days/months/years; TCI- total corneal infiltrate; NG-no growth; NA- not applicable; ND-not done; unid: unidentified; LFU-lost to follow up; Scr-C/S: Growth of fungus in culture from scraping samples; CB-C/S: Growth of fungus in culture from corneal button samples, Imp-Impression cytology.