Original Article

Coexistence of herpes simplex virus infection in microsporidial stromal keratitis associated with granulomatous inflammation

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Background: Microsporidial stromal keratitis poses several diagnostic challenges. Patients may present with corneal ulceration, marked stromal thinning, or even as a quite corneal scar. The presentation of microsporidial stromal keratitis commonly mimics viral keratitis. Microbiology scrapings are usually helpful; however, scraping and culture-negative cases pose a significant diagnostic dilemma. Histopathological examination is diagnostic but shows varying degree of inflammation, predominantly composed of polymorphonuclear leukocytes. Granulomatous inflammation, in microsporidial stromal keratitis, is never well described, and the authors in this article aim to describe the presence of granulomatous inflammation in microsporidial stromal keratitis, in patients with associated herpes simplex virus (HSV) keratitis. **Methods:** This was a retrospective and observational study conducted at a tertiary eye care center. **Results:** Of 263 patients who underwent therapeutic penetrating keratoplasty for infectious keratitis, during 2011–2013, seven patients were diagnosed as microsporidial stromal keratitis. Microsporidial spores could be demonstrated on microbiological scrapings in 5/7 (71%) of cases, but identified on histopathological examination and also confirmed on polymerase chain reaction (PCR) for microsporidium in 100% of cases. There was evidence of diffuse stromal necrosis with markedly severe degree of polymorphonuclear leukocytic infiltrates, with granulomatous inflammation in 42% of cases. Interestingly, these were positive for HSV-1 DNA on PCR. Review of medical records revealed much severe clinical presentations in patients with granulomatous inflammation, in comparison to cases without granulomatous inflammation. **Conclusions:** The authors hereby recommend that severe clinical presentation in patients with microsporidial stromal keratitis, markedly dense polymorphonuclear leukocytic infiltrates or the presence of granulomatous inflammation on the histopathological examination, should be investigated further for the presence of HSV-1 DNA for better patient management and good visual outcome.

Key words: Granulomatous inflammation, herpes simplex virus-1, *Microsporidia*, stromal keratitis, viral keratitis

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Ocular manifestations of microsporidial infections include the superficial punctate keratoconjunctivitis and microsporidial stromal keratitis. The former is a self-limiting disease as compared to microsporidial stromal keratitis, which shows poor response to medical therapy. Although recognized first in 1973 by Ashton and Wirasinha[1] a review of English literature revealed only 45 cases of microsporidial stromal keratitis.[1-11] Predisposing factors for stromal involvement by microsporidium are poorly understood. Clinical presentation of microsporidial stromal keratitis is nonspecific and may mimic viral keratitis, fungal keratitis, bacterial keratitis, or even a corneal scar. The diverse clinical presentation explains the variable morphological features on histopathology examination, the spectrum extending from severe to minimal degree of inflammation to scarred cornea. The clinical presentation may further be altered by treatment with steroids, antiviral, and antifungals. Furthermore, culture-negative samples and difficulty in differentiating *Microsporidia* from necrotic debris in routine stains explain the challenges and the need for an increased awareness in timely diagnoses of this infection by the ophthalmologist, microbiologist, and ocular pathologist. The presence of granulomatous inflammation on histopathological examination, in microsporidial stromal keratitis, is not previously well described in literature and the authors with this article report an interesting morphological observation of granulomatous inflammation in microsporidial stromal keratitis, which intrigued the authors to search for the cause of granulomatous inflammation and thus identified the presence of associated viral infection in these cases.

Case Description

In a retrospective observational study, approved by our Institutional Review Board, all the patients undergoing

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therapeutic penetrating keratoplasty (TPK) for infectious keratitis during the year 2011–2013 were analyzed, and 7/263 patients were found to be diagnosed as microsporidial stromal keratitis. The mean age of patients was 54.4 years (range 41–76 years). Of 7 patients, 5 were male, 3/7 were farmer by occupation and 2/7 had a history of trauma. None of them had a history of contact lens wear. Duration of symptoms varied from 1 to 9 months. Of 7 cases, 5 were diagnosed as microsporidial stromal keratitis on corneal scrapings, and all the seven cases demonstrated *Microsporidia* on histopathological examination. Of 7 cases, 3 (Group A: Case 1, 2, and 6) showed a severe degree of infiltration of polymorphonuclear leukocytes, posterior stroma showed granulomatous inflammation, and all the three cases of Group A were positive for herpes simplex virus-1 (HSV-1) DNA on polymerase chain reaction (PCR). Inflammation was less marked in cases 3, 4, 5, and 7 (Group B).

The Group B cases showed the absence of granulomatous inflammation and were negative for HSV-1 DNA. Detailed Clinical history and clinical examination findings [Figs. 1a, 2a and 2b; Cases 1, 4 and 5] of all the seven patients are tabulated in Table 1.

Clinical history of Case 1 (Group A)

Case 1

A 75-year-old male presented with chief complaints of severe pain, redness, watering, and blurring of vision in the left eye for 2 months. There was no history of trauma. At presentation, he was on 5% natamycin eye drop, 0.5% moxifloxacin eye drop, 3% acyclovir eye ointment, and 5% sodium chloride eye drop. The best-corrected visual acuity: the left eye was projection of light accurate with inaccurate projection of rays. Slit-lamp examination (SLE) showed diffuse congestion of conjunctiva, and cornea had an epithelial defect of 5.3 mm × 4.2 mm, central stromal thinning and deep stromal infiltrate of size 5.3 mm × 4.2 mm. There was an area of perforation measuring <1 mm, deep vessels in limbus all around, and a hypopyon of 1.4 mm [Fig. 1a]. Corneal scraping smears showed numerous microsporidial spores [Fig. 1b], and there was no growth on culture. He was advised topical 0.02% polyhexamethylene biguanide (PHMB) every hour, 0.02% chlorhexidine every hour, 1% atropine three times a day, and oral ketoconazole 200 mg twice a day. At 3-week follow-up, size of infiltrate increased and the condition worsened, TPK was performed, and the corneal button was sent to laboratory for microbiological and pathological investigations. Postoperatively, he was advised topical 0.02% PHMB every hour and 1% atropine three times a day. Tablet acyclovir 400 mg twice a day was advised for 4 months after the diagnosis of associated HSV keratitis was made. At the last visit, his vision [Table 1] was counting fingers at 2.5 m; graft was clear with grade 3 nuclear sclerosis.

Clinical history of Case 3 and Case 4 (Group B)

Case 3

A 41-year-old female, homemaker, presented with complaints of pain, watering, redness, itching, and blurring of vision for about 2 months in the left eye. She had a history of trauma with child's finger about 2 months back and was diagnosed to have microbial keratitis. At presentation, she was on 5% natamycin, 0.5% moxifloxacin, and tablet ketoconazole. Her best-corrected visual acuity was hand movement, and SLE

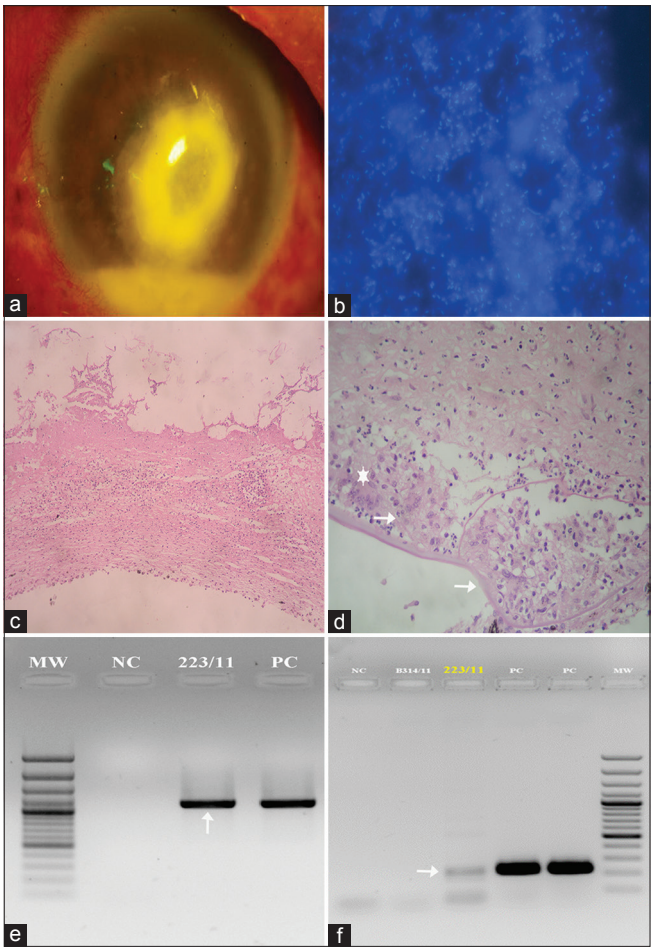

Figure 1: Case 1 (a) Slit-lamp picture showing central stromal infiltrate and hypopyon in diffuse illumination, (b) potassium hydroxide and calcofluor white wet mount of corneal scraping from a patient with microsporidial keratitis showing numerous microsporidial spores under a fluorescence microscope (original magnification × 400). (c) Low-magnification photomicrograph showing ulcerated corneal epithelium and surface covered by fibrin glue. There is diffuse stromal necrosis with loss of lamellar arrangement of stromal collagen. There is diffuse infiltration of polymorphonuclear leukocytes of severe degree admixed with debris material (H and E, ×100). (d) High-magnification photomicrograph shows granulomatous inflammation in the posterior stroma and along Descemet's membrane. Numerous histiocytes are seen with pale staining abundant cytoplasm and round to oval peripheral nuclei (arrow marked). Histiocytes are seen coalescing to form multinucleated giant cells (asterisk marked) (H and E, ×400). (e and f) Representative gel pictures of pan-microsporidial polymerase chain reaction (arrow marked in e) and Herpes simplex virus-1 polymerase chain reaction (arrow marked in f) showing amplification of 1200 base pair and 221 base pair products in patient sample number 223/11 respectively. NC: Negative control, PC: Positive control and MW– 100 base pair plus molecular weight DNA ladder

revealed a congested conjunctiva, central deep corneal stromal infiltrate measuring 3.8 mm in vertical and 3.7 mm in horizontal dimension, and stromal vascularization. Anterior chamber was deep with hypopyon of 4 mm. Corneal scraping smears and culture could not reveal any organisms. With clinical diagnosis of bacterial keratitis, gatifloxacin eye drops hourly was started. The condition worsened, and TPK was conducted after 2 weeks of initial visit, and the corneal button was sent

Table 1: Summary of clinical features, findings on corneal scrapings, polymerase chain reaction results, treatment and graft outcome in patients with histologically proven Microsporidial stromal keratitis

Age/ sex	DOS (months)	Occupation	Trauma	Medical treatment at presentation	Vision	SLE					Clinical diagnoses	Scraping/ culture	Medical/ surgical management	PCR *Microsporidia*/ HSV-1	DOP to TPK	Graft outcome/ VA at last visit
						Epithelial ulceration	Stromal thinning	Inflammatory infiltrate Inf	Hypopyon	Vascularization						
75/ male	2	Business	-	AB, AV, AF	PL, PR	+	+	+ (full thickness)	+	+	Fk/VK	*Microsporidia*/ negative	PHMB, Ktz/ TPK	+/+	3 weeks	Clear/CF 2.5 m
50/ male	9	Farmer	-	NA	PL, PR	+	+	+ (80%)	-	-	VK	*Microsporidia*/ negative	PHMB, Ktz/ TPK	+/+	5 days	Failed; endothelial decompensation/ CF 0.3 m
41/ female	2	Home maker	+ (child hand)	AB, AF, AV	HM	-	-	+ (anterior 2/3)	+	+	BK	Negative/ negative	Gatifloxacin/ TPK	+/-	2 weeks	Failed; endothelial decompensation/ PL, inaccurate. PR
47/ male	6	Farmer	-	Nil	20/50	-	-	+ (anterior 2/3)	-	-	VK	*Microsporidia*/ negative	PHMB, Ktz/ TPK	+/-	12 weeks	Clear/CF 1.0 m
46/ male	8	No occupation	+ (paddy leaf)	AF	20/400	+	-	+ (anterior 1/2)	-	+	FK	*Microsporidia*/ negative	PHMB, Ktz/ TPK	+/-	12 weeks	Clear/20/80
76/ male	1	Farmer	–	AB with steroids	HM	+	+	Posterior 2/3	+	-	BK	Negative/ negative	Gatifloxacin/ TPK	+/+	1 week	Clear/20/50
47/ female	6	Home maker	–	NA	CF	+	-	+ (anterior 1/2)	Trace+	-	FK	*Microsporidia*/ GPC	PHMB, albendazole and gatifloxacin/ TPK	+/-	3 days	Failed

- denotes: absent, + denotes: present. DOS: Duration of symptoms, DOP: Date of presentation, TPK: Therapeutic penetrating keratoplasty, SLE: Slit- lamp examination, AB: Anti-bacterial, AV: Anti-viral, AF: Anti-fungal, PL: Perception of light, PR: Projection of rays, HM: Hand movement, VA at pres: Visual acuity at presentation, PHMB: Polyhexamethylene biguanide, Ktz: Ketoconazole, NA: Not available, FK: Fungal keratitis, VK: Viral keratitis, BK: Bacterial keratitis, MK: Microbial keratitis, HM: Hand movement, GPC: Gram-positive cocci, CF: Counting fingers, HSV-1: Herpes simplex virus-1

to the laboratory. The patient was started on 0.02% PHMB eye drops 8 times/day and oral ketoconazole 200 mg twice a day as numerous *Microsporidia* were seen on histopathological examination. Due to endothelial decompensation, the graft failed and the patient underwent repeat graft. Subsequently, the patient had been lost to follow-up.

Case 4

A 47-year-old male, farmer, presented with chief complaints of pain, redness, watering, and decrease of vision in the right eye for 6 months. He had no history of trauma and was not on any medications. The best-corrected visual acuity was 20/50 and SLE showed intact corneal epithelium, central stromal scarring [Fig. 2a], and pigment deposits on endothelium. With clinical diagnoses of Viral Keratitis, the patient was started on tablet valaciclovir 1000 mg twice a day and prednisolone acetate eye drops four times a day in tapering doses. On 4-week follow-up, the patient showed partial improvement and then presented after 8 weeks with worsening of symptoms (in spite of treatment). The best-corrected visual acuity was counting fingers, and the patient had developed stromal infiltrates involving anterior two-thirds of the depth. There was no stromal thinning or vascularization. Corneal scraping revealed only two microsporidial spores and the patient was started on 0.02% PHMB eye drops 8 times/day and oral ketoconazole 200 mg twice a day. There was no growth on culture of corneal scrapings. The corneal scrapings were also collected for PCR for HSV-1 DNA and were negative. TPK was performed after 3 days since the depth of infiltrate increased and the corneal button was sent to the laboratory. The same medications were continued after TPK, and, subsequently, steroids were also added and tapered. The patient had a visual acuity of counting fingers at 1.0 m, and the graft was clear at his last visit.

Laboratory investigations and procedures

Microbiology and molecular diagnoses

Corneal scrapings were performed in all the cases at presentation and smeared on clean, presterilized glass slides for microscopic examination using KOH + CFW, Gram–stain, and/or modified ZN stain. Microsporidial spores were demonstrated in five (Case 1, 2, 4, 5, and 7) of these scrapings. The scraped material was inoculated onto 5% sheep blood agar or chocolate agar for bacterial and fungal culture and was negative in all cases except Case 7 which showed Gram-positive cocci on smears and bacterial growth on culture. Half corneal button (CB) received after TPK was inoculated into 5% SBA, CA, brain heart infusion broth, thioglycollate broth, Robertson's cooked-meat medium, Sabouraud's dextrose agar, potato dextrose agar, and nonnutrient agar with *Escherichia coli* overlay. There was no growth on culture of corneal button in all the cases, except in Case 7 which showed significant growth of *Streptococcus mitis*.

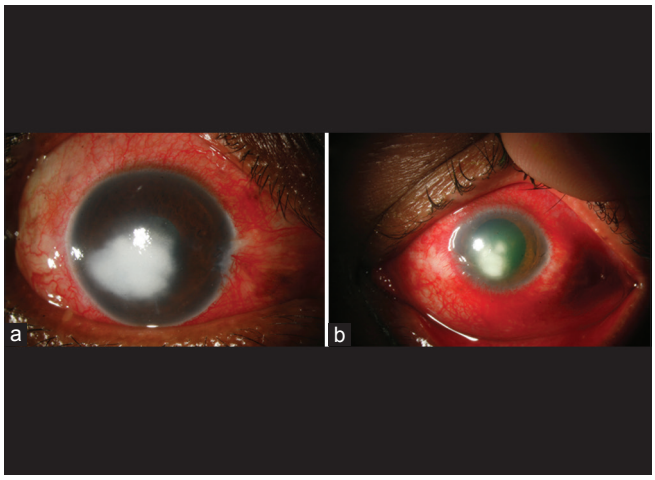

Figure 2: Slit-lamp picture showing (a, Case 4). Dense scar with surrounding clear stroma (b, Case 5). Stromal infiltrate in the mid-periphery with vessels from the limbus approaching towards the infiltrate

DNA was extracted from all the corneal button's using QIAamp DNA mini kit (QIAGEN GmbH, Hilden, Germany) using tissue protocol according to the manufacturer's recommendations. The extracted DNA was stored at −20°C till the PCR. All the samples were tested for the presence of microsporidial DNA by pan-microsporidial PCR targeting small subunit rRNA[12] and were found to be positive [Fig. 1e] in all cases. HSV-1 PCR[13] was initially performed only on cases showing granulomatous inflammation on the DNA extracted from formalin-fixed paraffin-embedded half corneal buttons. Amplification region for PCR was HSV-1 glycoprotein D gene with primer position being F: 19–43 and R: 218–239. Following PCR, the amplicon (221 bp) was resolved on a 1.5% agarose/TAE gel, visualized and recorded using ultraviolet gel documentation system (UVI Tec Ltd., Cambridge, UK) as described by our group earlier.[13] Fig. 1f shows a representative gel of PCR done for Case 1 (inked in yellow) with appropriate negative and positive controls.

Later, PCR for HSV-1 DNA was also done on Group B cases and was found to be negative. Positive results [Fig. 1f, Case 1] were observed only in the Group A cases. Pan-fungal PCR targeting internal transcribed spacer region[14] was also performed on DNA extracted from formalin-fixed paraffin-embedded tissues of Group A cases to rule out the fungal etiology and were found to be negative.

Histopathology findings

HPE of Group A cases showed epithelial ulceration, stromal thinning, diffuse stromal necrosis [Fig. 1c, Case 1], and patchy loss of fibrokeratocytic nuclei. There was diffuse to near diffuse, severe degree of infiltration of polymorphonuclear leukocytes (Case 2, Fig. 3a]. In addition, there was the presence of granulomatous inflammation along the posterior stroma and the anterior surface of Descemet's membrane (DM) [Figs. 1d, Case 1 and Fig. 3b, Case 2]. Granulomatous inflammation comprised histiocytes, epithelioid histiocytes, multinucleated giant cells, few lymphocytes, and polymorphonuclear leukocytes. These cases were carefully studied to analyze the presence of any foreign body and also for associated fungal infection or acanthamoeba.

In contrast, cases of Group B showed intact, although thinned to focally denuded corneal epithelium with continuous Bowman's membrane. There was the absence of significant stromal necrosis or thinning, except Case 5 which showed patchy stromal necrosis [Fig. 4a, Case 4]. There was minimal, mild to moderate patchy infiltration of polymorphonuclear leukocytes [Fig. 4b, Case 4], more significant fibrosis, and absence of granulomatous inflammation. Numerous spores of *Microsporidia* were observed in all the cases, these appeared

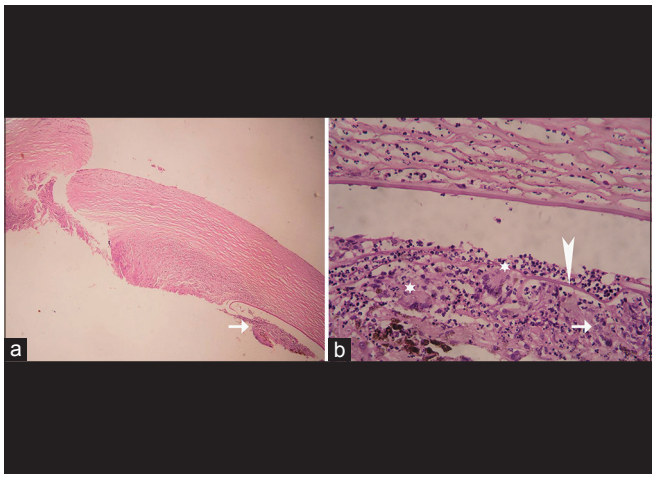

Figure 3: (Case 2) (a) Photomicrograph shows perforated corneal ulcer with stromal necrosis, and anterior and posterior stromal polymorphonuclear leukocytes infiltrate. Descemet's membrane is fragmented and folded. The inflammatory infiltrate (arrow marked) is seen adjacent to folded Descemet's membrane (H and E, ×100). (b) High-magnification photomicrograph shows fragmented Descemet's membrane (arrowhead marked) with granulomatous inflammation. There is infiltration of numerous histiocytes (arrow marked), multinucleated giant cells (asterisk marked) and polymorphonuclear leukocytes. Scattered uveal pigment is also present (Periodic acid–Schiff's stain × 400)

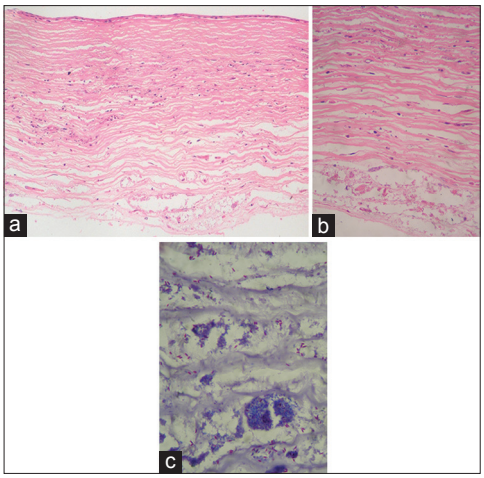

Figure 4: (Case 4) (a) Low-magnification photomicrograph shows intact corneal epithelium and continuous Bowman's membrane. Anterior stroma shows fibrosis with mild polymorphonuclear leukocytes infiltrate in the middle 1/3rd (H and E, ×100). (Case 4) (b) High-magnification photomicrograph shows stromal fibrosis and patchy myofibroblastic transformation of fibrokeratocytic nuclei. There are numerous pale basophilic to amphophilic spores (appears like debris) as compact clusters and dispersed singly. Also noted are the spores aligned along the stroma lamellae (arrows marked) (H and E, ×400). (c) Photomicrograph shows acid-fast and nonacid-fast, oval to slightly elongated microsporidial spores (Ziehl–Neelsen stain, ×1000)

pale blue to amphophilic staining oval to round bodies in hematoxylin and eosin stain [Fig. 4b], measuring approximately 3–5 microns in length and 2–3 microns in thickness. These spores were found as small aggregates and dispersed singly, predominantly in the interstromal spaces or aligned along the stromal lamellae and also in the region of stromal necrosis. Their distribution and number had an association with the distribution of the stromal polymorphonuclear leukocytic infiltrate, spores being higher in number, in areas which lacked inflammatory infiltrate. There was the absence of spores in the foci of granulomatous infiltrate or in the cytoplasm of the histiocytes or giant cells. On staining with Periodic acid–Schiff's (PAS) stain, these spores appeared pale basophilic to magenta pink with basophilic polar band. However, they were not easily spotted on PAS stain; therefore, Grocott's Methenamine Silver (GMS) and modified ZN (1% H_2SO_4) were carried out in all the cases. GMS stain stained these spores as brownish against the green background and the polar band was better appreciated. The spores were best highlighted on modified ZN stain, which stained them red with admixed partially stained and unstained spores which appeared blue [Fig. 4c]. Polar band was appreciated in few spores when stained with modified ZN (1% H_2SO_4) stain.

Discussion

Diverse clinical presentation in microsporidial stromal keratitis and thus variation in histopathology findings is well understood; however, to the best of our knowledge, literature lacks explicit observation of granulomatous inflammation in microsporidial stromal keratitis. Our cases of Group A characteristically presented as perforated corneal ulcer, morphologically were diagnosed as necrotizing stromal keratitis of microsporidial etiology, and also displayed granulomatous inflammation along the Descemet's membrane. On the contrary, the corneal tissues of Group B patients showed minimal to mild degree of inflammation, no stromal thinning, and absence of granulomatous inflammation. Vascularization was observed in both groups. Classical resemblance of clinical and morphological findings of cases of Group A, with viral keratitis, intrigued the authors to investigate for associated viral etiology. Ashton and Wirasinha reported the first case of microsporidial stromal keratitis in 1973 with detailed documentation of histopathology findings.[1] They reported the presence of predominantly necrotic stroma, acute inflammatory infiltrate, and presence of numerous microsporidial spores in the deep stroma. Interestingly, they also reported the presence of numerous giant cells and pigment granules on inner surface of Descemet's membrane with the absence of microsporidial spores in these giant cells. Pinnolis *et al*. in 1981 also reported the presence of granulomatous inflammation along the Descemet's in a perforated corneal ulcer with markedly necrotic stroma.[2] However, these cases reported in literature were not investigated for viral etiology.[1,2] Diffusion of toxins released by the proliferating parasites was speculated as the possible cause for granulomatous inflammation; however, toxins as the cause for granulomatous inflammation are less convincing since 17 of 21 cases of microsporidial stromal keratitis described in literature with morphological findings did not report the presence of granulomatous inflammation.[1-11]

Granulomatous inflammation in herpetic stromal keratitis is reported to have a frequency of 25%, commonly observed in patients presenting with ulcerative necrotizing stromal keratitis in comparison with nonulcerative/scarring stromal keratitis, with localization along the DM in 94% of the cases,[15] attributed to poorly understood alteration in the antigenicity of DM.[16] It was interesting to note our Group A cases had

similar morphological findings as compared to Group B. The limitation of our study was that we could not supplement our HSV-1 PCR results with immunofluorescence assay, since the representative formalin-fixed paraffin-embedded tissue was insufficient after extracting DNA from it, and the authors had not preserved any fresh sections; however, a different study from our Institute has reported 100% sensitivity of HSV PCR.[13] The possibility of false-positive results due to contamination was overcome by taking care that negative control added to all the runs was negative.

Vascularization was observed clinically in 3/7 cases, of which only one case belonged to Group A; thus, the presence or absence of chronic granulomatous inflammation or immune inflammatory reaction could not be significantly or solely attributed to vascularization. Our cases of both groups did not demonstrate any significant difference in the functional outcome of grafts. This could be because that Group A patients, besides treatment with steroids, were also treated with systemic antivirals for duration of 4 months, started at 1st week postoperative visit, as they were diagnosed to have associated HSV keratitis. Systemic prophylaxis for viral recurrence and graft rejection by well-adopted local steroid therapy when instituted with close follow-ups leads to similar functional outcomes of grafts in individuals with keratitis of herpetic or nonherpetic origin.[17]

There has been an association of microsporidium with viral infections; the first case of MSK was reported in 1990 in an HIV-seropositive patient.[18] In the late 1990s and the early part of the 21st century, microsporidial keratoconjunctivitis was reported from immunocompetent individuals also. Recently, the coexistence of *Microsporidia* and adenovirus in the corneal lesions of patients with epidemic keratoconjunctivitis was also reported.[19] However, to the best of our knowledge, this is the first study of coexistence of HSV infection and microsporidial stromal keratitis in patients presenting with necrotizing stromal keratitis and granulomatous inflammation.

Conclusions

We recommend that patients presenting to Cornea clinic with almost full-thickness corneal infiltrates and stromal thinning, subsequently diagnosed as microsporidial stromal keratitis either on scraping or are undiagnosed and are culture negative, should be carefully searched for microsporidial spores on histopathology. Cases with granulomatous inflammation on histopathology examination or severe necrotizing inflammation should also be investigated for associated viral infection, by eliciting PCR for HSV-1 DNA from corneal scrapings. This can also be performed on formalin-fixed paraffin-embedded tissue if fresh corneal tissue is not available for PCR. These patients need careful follow-up and therapeutic strategies to prevent graft rejection or viral recurrence.

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Conflicts of interest

There are no conflicts of interest.

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