MYCOLOGY

An in-vitro study of the sterol content and toxin production of *Fusarium* isolates from mycotic keratitis

S. K. RAZA, A. I. MALLETT*, S. A. HOWELL and P. A. THOMAS†

St John's Institute of Dermatology, UMDS, University of London, St Thomas' Hospital, London SE1 7EH and

† Institute of Ophthalmology, Joseph Eye Hospital, Tiruchirapalli 620 001, India

Summary. Isolates of *Fusarium* species from 18 patients with keratomycosis were examined for their C-29 and C-31 sterol content and for their capacity to synthesise mycotoxins. All isolates were resistant to azole antifungal agents *in vitro* and the sterol contents were indistinguishable. In-vitro toxin production was monitored by gas chromatography–mass spectrometry; 13 isolates produced nivalenol, six produced deoxynivalenol, nine gave T-2 toxin and two showed the presence of diacetoxyscirpenol at different time intervals. However, neither sterol content nor toxin production *in vitro* appeared to be related to the severity of infections observed in patients.

Introduction

Mycotic keratitis is a suppurative, and frequently ulcerative, fungal infection of the cornea which normally follows trauma. Identification of the infecting agent relies on the examination of cultures grown from corneal scrapings and biopsy samples. In tropical and subtropical regions, *Fusarium solani* and *F. oxysporum* are the commonest causes of keratitis. Infection with these fungi is characterised by rapid corneal sloughing and marked visual loss and may be difficult to treat.¹

The response of mycotic keratitis to antifungal therapy has been reported to be influenced by the type of fungus and the severity of the infection on initial presentation, and not by the duration of the infection, history of prior treatment or the age and sex of the patient.² Antifungal susceptibility of the infecting fungus may also influence therapeutic success, and in-vitro assays for isolates of *Fusarium* have shown varying susceptibility to amphotericin B, miconazole and ketoconazole; nearly all isolates are resistant to 5-flucytosine.³

Most of the *Fusarium* spp. isolates from patients with mycotic keratitis have a worldwide distribution in soil and have been found in both plants and animals. Cuero⁴ examined *F. solani* isolates recovered from keratitis of human eyes and from the environment and found both groups to exhibit the same pathogenicity in tests on young beans, corn and tomato plants, and to evoke an erythematous reaction when tested in rabbit

eyes. The substance causing this reaction in an eye test was described as an ultraviolet absorbing extracellular substance, but no further identification was made. The potential and identification of toxins produced by *Fusarium* spp. isolated from ocular infections of man have not been fully explored.

Interest in the toxins of this genus arises chiefly as a result of its role as a plant pathogen,⁵ and as a major cause of a range of animal and human disorders^{6–8} resulting from ingestion of toxins produced in fungus-contaminated grains. Infections caused by *Fusarium* spp. include onychomycosis, mycotic keratitis, leg ulcers and granulomatous skin lesions and they are being increasingly reported as causes of infection in immunocompromised patients.⁹ They also infect burn wounds which may lead to dissemination.¹⁰ In a review of the role of fusarium infections in cancer patients, Anaissie and colleagues¹¹ remarked that an important feature of this genus is its ability to cause myelosuppression through toxin production, and suggested that although an association between fusarium infection and prolonged myelosuppression has not been proved, it should be considered.

In this investigation the sterol composition and the ability to produce toxins *in vitro* was examined in 18 *Fusarium* isolates from patients with mycotic keratitis who responded with various degrees of success to antifungal therapy.

Table 1. Clinical response to antifungal therapy and fungal isolates recovered from patients with severe or non-severe keratitis

Response to therapy	Isolates from severe infection	Isolates from non-severe infection
Poor	Fs 36, 53, 57, 58, 248, 284 NID 214	...
Excellent	Fs 68, 242, 243, Fox 272	Fs 107, 173, 189, Fsp 60, 157, 174, 248

Fs, *Fusarium solani*; Fsp, *Fusarium* species; Fox, *F. oxysporum*; NID, not identified.

Materials and methods

Clinical details

Corneal scrapings were taken from patients with clinical symptoms of mycotic keratitis attending the Cornea Clinic, Institute of Ophthalmology, Joseph Eye Hospital, Tiruchirapalli, India. The collection of specimens and the method of culture, with the classification of the corneal ulcers as severe or non-severe before treatment, have been described elsewhere.¹² Patients were given an oral treatment regimen of either ketoconazole 600 mg/day or itraconazole 200 mg/day. The topical therapy used included a 1% solution of azole (ketoconazole, itraconazole, saperconazole or econazole) or a 5% solution of natamycin which was administered hourly until the ulcer healed. The duration of treatment ranged from 8 to 44 days, and the response of each patient to treatment was graded as excellent (mycological and clinical cure), moderate (mycological cure with clinical improvement) or poor (mycological and clinical failure).

Fungal isolates

The isolates were identified on the basis of morphological and microscopic appearance. Twelve isolates were identified as *F. solani*, four as *Fusarium* spp., one as *F. oxysporum* and one isolate failed to spore adequately for recognition (not identified; NID 214). Spores and mycelial fragments from the cultures were stored in distilled water until required.

Susceptibility to antifungal agents

Inhibitory concentrations of azole antifungal agents were measured by the agar dilution method of Shadomy *et al.*,¹³ on Kimmig agar.

Sterol composition

Fungal sterols were extracted and analysed as described elsewhere.¹⁴ Briefly, after growth in Sabouraud dextrose broth, the sterols were extracted from dried mycelia by alkaline saponification, deriv-

Compound	R ¹	R ²	R ³	R ⁴	R ⁵
T-2	OH	OAc	OAc	H	i-C ₄ H ₉ COO
DAS	OH	OAc	OAc	H	H
DON	OH	H	OH	OH	=O
NIVA	OH	OH	OH	OH	=O
FUS-X	OH	OAc	OH	OH	=O

Figure. Structures of mycotoxins referred to in the text.

atised to form either trimethylsilyl (TMS), or *tert*-butyldimethylsilyl (TBDMS) ethers for identification by gas chromatography-mass spectrometry (GC-MS). Separations were performed on an SE-30 capillary column (12 m × 0.32 mm; Thames Chromatography, Maidenhead) with a temperature programme from 175°C to 300°C, on a Hewlett-Packard 5890 GC directly coupled to the source of a VG Mass-Lab Trio-2 quadrupole MS (Fisons Instruments Ltd, Manchester).

Toxin production

The isolation and identification of the six fusarium toxins, whose structures are given in the figure, have been described previously.¹⁵ In outline, the isolates were grown on YES agar (yeast extract 20 g/L, sucrose 150 g/L, agar no. 1 20 g/L) at 25°C in the dark. An agar plug was removed from the centre of the colony on days 14, 20, 35 and 50 after inoculation and placed directly on to a pre-coated silica gel TLC plate (BDH, Dagenham, Essex) for 30 s. The plates were developed in toluene:ethyl acetate:formic acid 90% (5:4:1, v:v:v) and the toxins were detected with spray

reagents and ultraviolet light. The regions of the TLC plate corresponding to the R_f values of standard toxins were combined, eluted through BondElut silica cartridges (Jones Chromatography, Mid Glamorgan) and derivatised with *N,O*-bis-trimethyl-silyltrifluoroacetamide (BSTFA) containing trimethylchlorosilane (TMCS) (Pierce and Warriner, Cheshire) 1% to form trimethylsilyl ethers. GC-MS analysis of the derivatised toxins was with a Restek Rtx-1 capillary column (30 m $\times$ 0.32 mm; Thames Chromatography) with a temperature programme of 150°C for 2 min followed by a gradient to 300°C at 20°C/min. The column was installed in a Hewlett-Packard 5890 GC directly coupled to the source of a VG 70 SEQ tandem hybrid MS (Fisons Instruments Ltd). Identification was achieved by comparison with standard toxins (Sigma) by use of selected ion recording (SIR) and multiple reaction monitoring (MRM) with GC-MS and GC-MS-MS respectively.

Results

Treatment outcome

Eighteen patients with mycotic keratitis due to *Fusarium* spp. were treated with antifungal agents (azoles and natamycin) orally or topically, or both, and the fungal isolates were tested for the minimum inhibitory concentration (MIC) to these compounds. Approximately one-third of the patients failed to respond to treatment (table I); these were classed as having severe infections caused by *F. solani* (six patients) and the unidentified isolate 214 (one patient). An excellent response to treatment was recorded for the remaining 11 patients; four presented with severe infections (three with *F. solani* and one with *F. oxysporum*), and seven were classified as non-severe (three with *F. solani* and four with *Fusarium* spp.).

Sterol composition

Although the isolates demonstrated no susceptibility to azoles *in vitro* (MIC 64–128 mg/L) this did not reflect the response *in vivo*, since two-thirds of the patients improved significantly. The antifungal susceptibilities could not be linked to sterol composition. The major sterol detected was ergosterol, with episterol in smaller quantities and trace amounts of minor components, including cholesterol, brassicasterol, fecosterol, campesterol and sitosterol. Sterol intermediates such as lanosterol were detected either in trace amounts or not at all. There was no significant difference in the sterol composition of isolates between patients who responded or who failed to respond to therapy.

Toxin production

Toxin production was monitored semi-quantitatively at intervals over a 50-day growth period for 17 of the fungal isolates (table II). Thirteen of the isolates

Table II. Mycotoxin production in *Fusarium* isolates: mass spectrometric data

Isolate	Days	DON	DAS	NIVA	T-2
Fs 57	14	—	—	+	—
	20	—	—	+++	—
	35	—	—	+++	—
	50	—	—	+	—
Fs 68	14	—	—	+	—
	20	—	—	+++	—
	35	—	—	+++	—
	50	—	—	++	—
Fs 107	14	—	—	++	—
	20	—	—	+++	—
	35	—	—	+++	—
	50	—	—	+	—
Fsp 157	14	—	—	+	—
	20	—	—	+++	—
	35	—	—	+++	—
	50	—	—	++	+
Fs 189	14	—	—	+	—
	20	—	—	+++	—
	35	—	—	+++	+
	50	—	—	+	—
Fsp 174	14	—	—	—	+
	20	—	—	—	—
	35	—	—	—	—
	50	—	—	—	—
Fs 242	14	—	—	—	+
	20	—	—	—	—
	35	—	—	—	—
	50	—	—	—	—
Fs 243	14	—	—	++	—
	20	—	—	+++	—
	35	—	—	+++	—
	50	+	—	+	++
Fsp 248	14	—	—	+	—
	20	—	—	+++	—
	35	—	—	+++	—
	50	—	—	++	—
NID 214	14	—	—	—	+
	20	—	—	—	—
	35	—	—	—	—
	50	—	—	—	—
Fs 53	14	—	—	+	—
	20	+	—	+++	—
	35	—	—	+	—
	50	—	+	+	+
Fs 58	14	—	—	+	—
	20	—	—	+++	—
	35	—	+	+++	—
	50	+	—	++	++
Fsp 60	14	—	—	+	—
	20	—	—	+++	—
	35	—	+	+++	—
	50	—	—	+	—
Fs 173	14	—	—	—	+
	20	—	—	—	—
	35	—	—	—	—
	50	—	—	—	—
Fs 248	14	—	—	+	—
	20	—	—	+++	—
	35	—	—	+++	—
	50	+	—	++	++
Fox 272	14	—	—	+	—
	20	—	—	+++	—

Fs, *F. solani*; Fox, *F. oxysporum*; Fsp, *Fusarium* species; NID, not identified; —, not detected; +, *30–45%; ++, *45–75%; +++, *75–100%.

*Relative absolute intensities of SIR and MRM signals in ion chromatograms.

Table II. (cont.)

Isolate	Days	DON	DAS	NIVA	T-2
Fs 284	35	+	—	+++	—
	50	—	—	+	—
	14	—	—	++	—
	20	—	—	+++	—
	35	—	—	+++	—
	50	—	—	+	++

showed a gradual increase in production of nivalenol from day 14 to day 35, after which its concentration declined. Some of these isolates also produced deoxynivalenol (six isolates), diacetoxyscirpenol (two) and T-2 toxin (nine) at different times. No evidence was found for the production of fusarenone-X or zearalenone. The ability of the isolates to produce one or more toxins did not correlate with either the severity of infection on initial presentation or with the response to treatment (tables I and II).

Discussion

The observation that, of the 11 patients with severe infections, four improved but seven failed to respond to the antifungal therapy, suggested that there might have been further factors influencing the result of treatment. This may have been a characteristic of the infecting organisms. Measurement of the MICs of the fungal isolates did not reflect the in-vivo response to therapy. However, testing antifungal susceptibility to azoles is known to be difficult to perform and it is not unusual to obtain an MIC that is above the drug concentration that can be achieved in the patient.¹⁶ One of the mechanisms of action of azole antifungals is to inhibit sterol biosynthetic pathways by preventing C₁₄ demethylation of lanosterol. This leads to the accumulation of sterically bulky sterols in the membrane, causing a reduction in membrane integrity and disruption of membrane-bound enzymes. In *Candida* spp. and *C. albicans*, altered sterol compositions have been associated with reduced azole susceptibility.^{17,18} However, since the sterol content of the *Fusarium* isolates from the patients with keratitis that responded to treatment were indistinguishable from those that failed to respond to therapy, it would appear that treatment success or failure could not be attributed simply to the sensitivity of the isolates to antifungal agents.

Several toxins produced by *Fusarium* spp. associated with contaminated foods and grains have been identified,^{19–21} including T-2 toxin (T-2), diacetoxyscirpenol (DAS), deoxynivalenol (DON), nivalenol (NIVA), fusarenone-X (FUS-X) and zearalenone (ZEA) (figure). Amongst these, only T-2, DAS and FUS-X have been examined for dermal toxicity,²² the major histological change being an increase in the stratum malpighii with other changes including mild to moderate degeneration of fibroblasts and cellular infiltration in the corium of the skin. Four toxins were produced by our isolates in culture—T-2, DAS, DON and NIVA; neither ZEA nor FUS-X was detected (data not shown). However, toxin production *in vitro* did not relate to the clinical classification of severity of infection or treatment outcome. Details of the time that elapsed between the initial eye injury and the appearance of clinical keratitis in the patients were not available and hence could not be correlated with the time scale for maximal production of NIVA observed in this study. However, previous studies have shown that the duration of infection did not influence the response to antifungal therapy.¹

The YES growth medium used here was originally developed to provide a TLC classification system for *Penicillium* spp. based on mycotoxin and secondary metabolite production,²³ and later was used to demonstrate toxin production by a variety of *Fusarium* spp.²⁴ Such a medium would provide optimal nutrition for toxin production, a situation that probably would not exist *in vivo*. The present study has provided valuable information concerning which toxins can be produced by *Fusarium* spp. infecting man and the sensitivity and specificity of mass spectrometry will make it an ideal tool for the detection of targeted toxins *in vivo*.

In conclusion, sterol composition and the ability to produce toxins *in vitro* were examined as possible contributors to the pathology of keratitis due to *Fusarium* spp. described in 18 patients. Differences in clinical outcome could not be explained by the results obtained *in vitro* in these studies but aspects which we have not investigated here could influence the development and severity of keratitis. These include the ability of these fungi to produce various enzymes which could damage tissue and facilitate invasion,^{1,25} and factors dependent on the individual patient such as hypersensitivity reactions to the fungal material or to toxins released from it, or to underlying immune deficiencies. Clearly the aetiology of this disease is complex.

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