

Conjunctival fungal flora of the clinically normal pony eye : Including *Nocardia* and *Streptomyces* species

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(Received Feb 11, 1998)

임상적으로 건강한 조랑말의 결막 진균총 조사 : *Nocardia* 종 및 *Streptomyces* 종을 포함하여

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(1998년 2월 11일 접수)

초 록 : 영국 Hertfordshire의 두지역에서 사육되고 있는 건강한 조랑말의 결막내 상존하고 있는 진균총의 분포도를 조사 비교하고자 36두 조랑말의 양안(총 72안)의 상·하결막낭에서 시료를 채취하여 진균을 분리 동정하였다. 총 76.4%의 안구에서 진균이 발견되었으며, 13종이 분리되었다. 전체적으로 가장 많이 분리된 진균은 *Aspergillus* spp(27.8%), *Nocardia* spp(25.0%), *Cladosporium* spp(19.4%), *Penicillium* spp(9.7%) 및 *Streptomyces* spp(9.7%) 순이었다. 1지역에서는 *Nocardia* spp와 *Cladosporium* spp가 가장 많이 분리된 반면, 2지역에서는 *Aspergillus* spp가 가장 많이 분리되었다. 상·하결막낭내 진균분리율의 차이는 상결막낭에서보다는 하결막낭내에서 진균이 유의성 있게 많이 분리되었다($p < 0.05$). 시료채취시에 사용된 swab의 상태에 따른 비교에서 건조된 swab과 젖은 swab 간에는 통계적 유의성이 없었다.

Key words : conjunctiva, eye, fungal flora, equine keratomycosis, pony.

Introduction

Keratomycosis in the horse is not uncommon in practice and appears to be increasing in frequency of recognition¹⁻³. The common causes of keratomycosis may be related to the injudicious use of topical or systemic corticosteroids medication and spontaneous infection related or organic trauma^{4,5}. Topical corticosteroids interfere with nonspecific and cell-mediated immune responses of the anterior segment and pre-corneal tear film, establishing a favourable situation for fungal overgrowth^{3,6}. Most keratopathogenic fungi in the horse are opportunistic pathogens which exist as part of the commensal flora harboured in the conjunctival fornices, lid margins and proximal nasolacrimal drainage system of the normal healthy eye⁷. The majority of cases of mycotic keratitis are not primary, but occur secondary to corneal trauma that results from corneal abrasion with some type of plant material⁸⁻¹⁰. Injuries of the cornea and the conjunctiva of the horse are relatively common^{11,12}, as they have relatively large, prominent eyes which are susceptible to damage by straw or dirt from the environment¹⁰. Keratomycosis is therefore observed more frequently in the horse than in other domestic species⁹. Riis⁵ found that on several occasions fungi isolated on control plates exposed to the environment were the same as those isolated from the horse's eye. He suggested that fungi occurring in the outer eye were probably transient or at least related to random seeding from the environment. It is often difficult to determine the significance of fungus that is not a primary pathogen when it is isolated from a clinical specimen¹³. A knowledge of the prevent fungal flora may aid our understanding of the epidemiology of equine keratomycosis and may assist the clinical pathologist and veterinarian in diagnosis and in choosing an appropriate agent for initial therapy¹⁴. However, literature regarding the mycotic flora of the equine eye in the UK is extremely limited, particularly in the pony.

A number of researchers in the USA have published reports on the normal fungal flora in the horse. Lundvall¹¹ recovered mainly *Aspergillus* spp, *Penicillium* spp, *Absidia* spp, *Fusarium* spp and *Cladosporium* spp in Iowa from 250

conjunctival swabs. Eastwood¹⁵ attempted fungus isolation from one eye of each of 216 horses in Iowa. 399 organisms were recovered from 212 eyes. These included 111 different species from 28 different genera. The fungi most commonly identified in this study were *Aspergillus* spp, *Alternaria* spp, *Penicillium* spp, *Fusarium* spp and *Absidia* spp. The fungal flora of the normal equine conjunctiva was further investigated at Cornell, New York by Riis⁵ who recovered 44 fungal isolates from 50 cultures. The main isolates in this study were *Aspergillus* spp and *Cladosporium* spp. Whitely *et al*¹⁶ reported that few fungi were isolated in a study of 50 eyes in Wisconsin and suggested that this was due to the influence of environmental and seasonal factors on microbial populations of the external eye of the horse. An investigation conducted by Samuelson *et al*¹⁷ isolated fungi, of which species of *Aspergillus*, *Penicillium*, *Alternaria* and *Cladosporium* were the common isolates, from 95% of the horses sampled in Florida. The differences in the prevalence of normal fungal flora between these studies may be associated with environmental factors, such as geographical location, climate, season, husbandry, source and type of bedding, feed, and individual habitat¹⁵⁻¹⁷.

Other reports have concerned the prevalence of mycotic keratitis in diseased horses. The organisms most frequently isolated from cases of equine mycotic keratitis included *Aspergillus* spp, *Fusarium* spp, *Penicillium* spp and the Phycomyces group^{1,3,4,9,14,18,19}. Peiffer⁹ diagnosed keratomycosis on the basis of clinical findings in 10% of horses referred for anterior segment disease in North Carolina, USA. McLaughlin *et al*¹⁴ surveyed clinical swabs from 123 eyes of 123 horses with external ocular diseases in Illinois. Fungi were isolated in 4.8% of the cases. Moore *et al*¹⁸ recovered fungi from 39%(15 of 38) horses with clinical ulcerative keratitis in Missouri. Barton¹, in a retrospective study of equine keratomycosis at the University of Georgia between 1980 and 1990, found one third of the cases of keratitis in horses were caused by fungal infection. Grahn *et al*¹⁹ reported that 7%(23/313 horses) of the total equine ophthalmic admissions received a diagnosis of keratomycosis in Ontario. Hamilton *et al*²⁰ diagnosed fungal keratitis in 36% of 11 corneal stromal abscesses by his-

topathology at the Auburn University and the Ohio State University veterinary teaching hospitals.

Thus, as the incidence of equine keratomycosis in the USA at least in high, antifungal therapy should be instituted immediately whilst awaiting laboratory confirmation if keratomycosis is suspected. Antifungal therapy will not harm the cornea, even if the diagnosis is wrong²¹. Peiffer⁹ and Beech *et al*⁴ suggested that when presented with deep corneal lesions, if the history and clinical findings are consistent with a diagnosis of mycotic keratitis, antifungal therapy should be initiated immediately, even if scrapings are negative.

Conjunctival culturing techniques vary with the investigator and clinician. Hacker *et al*²² stated that the wet swab culture technique was superior to the dry swab technique for conjunctival bacteria in the dog. Most investigators have used the wet swab technique for culturing the conjunctival fungi in the horse^{1-3,9,10,14,16,18}.

The aims of this study are to investigate the prevalence of fungal isolates from the conjunctival sacs of normal ponies in Hertfordshire, UK; to compare the conjunctival fungal flora between two different herds, and the distribution of fungi between the upper conjunctival sac vs. the lower conjunctival sac; and also to compare the efficiency of two different methods of obtaining conjunctival swabs for fungal culture.

Materials and Methods

Seventy-two eyes (thirty-six ponies) were sampled in this study; forty eyes in herd 1 and thirty-two eyes in herd 2. All the ponies included in this study belonged to the Royal Veterinary College, University of London. The ponies sampled included 22 females and 14 males, of ages from 2 to 23 years old; twenty-five were aged between 2 and 10 years and eleven were aged between 11 to 23 years. 15 New Forest, 4 Welsh Mountain, 3 Exmoor, 2 Fell and 12 cross breed ponies which were kept outdoors in paddocks were used. Prior to sampling all eyes were examined using magnification and focal illumination. Only subjects with no clinical evidence or recent history of external ocular in-

flammation or disease were selected.

The ponies were swabbed under physical restraint without the use of local or topical anaesthetics. The eyelids were manually opened, and gentle digital pressure close to the eyelid margin used to expose the conjunctival sac. Specimens were obtained by rotating the swab directly within the conjunctival sac taking care to avoid contact between the eyelid margin or lashes and the swab. Sampling was carried out during the period January to February. Two samples were obtained from each eye, one from the upper conjunctival sac and one from the lower conjunctival sac.

Four different sampling methods were used in this study in order to compare dry swab technique with wet swab: - dry swab for both conjunctival sacs; wet swab for both conjunctival sacs; dry swab for upper conjunctival sac and wet swab for lower; and wet swab for upper conjunctival sac and dry swab for lower. The wet swab was moistened with sterile saline before sampling. A pure viscose swab (Technical Service Consultants LTD, Lancashire, UK), not cotton, was used, since cotton has natural fungal inhibitors²¹.

The material obtained was immediately taken to the laboratory and transferred directly to sterile Sabouraud's dextrose agar (Oxoid[®], Unipath LTD, Hampshire, UK) without refrigeration.

These were incubated unsealed at room temperature (26°C). Plates were not discarded as 'negative' until they had been kept for at least 4 weeks with no fungal growth.

Each plate was divided into 3 equal sections for specimens from the upper and lower conjunctival sac and control. Visible streaks were made on the agar surface with the inoculating swab and fungal growth away from the streaks was regarded as contamination. The morphologic features and appearance of isolates were noted and fungal identification was accomplished microscopically by standard morphological criteria¹³. To encourage sporangium formation some unidentified fungi were subcultured from the Sabouraud's dextrose agar onto Malt extract agar (Oxoid[®]) and incubated again at 26°C and observed for growth. Microscopic slides were viewed from mounts in lactophenol cotton blue.

If a fungal species was isolated from both upper and low-

er conjunctival sacs in the same eye, it was regarded as a single isolate for that eye.

Data from the wet vs. dry swab techniques and upper vs. lower conjunctival sac was analysed with the Wilcoxon matched-pairs test to determine the difference of total numbers of isolates. When the numbers of isolates were equal, the data was excluded. A confidence interval of $\alpha = 0.05$ was used.

Results

Fungi were isolated from conjunctival sacs of 55 of the 72 eyes(76.4%). A total of 13 different fungi were identified but 13 isolates(18.1%) remained unidentified because of failure to sporulate. The most common fungi were *Aspergillus* spp, *Nocardia* spp, *Cladosporium* spp, *Penicillium* spp and *Streptomyces* spp, which accounted for 27.8%, 25.0%, 19.4%, 9.7% and 9.7%, respectively of the total number of isolates(Table 1, Fig 1). Other fungi isolated were

Acremonium spp, *Paecilomyces* spp, *Fusarium* spp, *Exophiala* spp, *Trichosporon* spp, *Tritirhachium* spp and *Verticillium* spp. Most fungal organisms grew within 1 week of inoculation.

In herd 1, fungi were isolated from 31 of the 40 eyes(77.5%) with *Nocardia* spp(35.0%) and *Cladosporium* spp(27.5%) as the predominant isolates(Table 2). In herd 2, fungi were isolated from 24 of the 32 eyes(75.0%), with *Aspergillus* spp(59.4%) as the predominant isolate(Table 3).

A total of 12 and 9 different fungi were identified in herds 1 and 2, respectively. Other fungi isolated in herd 1 were *Penicillium* spp, *Streptomyces* spp, *Acremonium* spp, *Paecilomyces* spp, *Aspergillus* spp, *Exophiala* spp, *Fusarium* spp, *Sepedonium* spp, *Trichosporon* spp and *Verticillium* spp(Table 2). In herd 2, other fungi isolated included species of *Nocardia*, *Cladosporium*, *Penicillium*, *Streptomyces*, *Acremonium*, *Fusarium*, *Paecilomyces* and *Tritirhachium* (Table 3). Unidentified fungi comprised 17.5% and 18.8% of all isolates, in herds 1 and 2, respectively

Table 1. Fungal isolates from the conjunctival sacs of normal ponies(72 eyes)

Fungus	(% of eyes sampled)								
	Wet Swab		Dry Swab		TUCS	TLCS	TWS	TDS	TE
	UCS	LCS	UCS	LCS					
<i>Acremonium</i> spp	1.4	1.4	1.4	1.4	2.8	2.8	2.8	2.8	5.6
<i>Aspergillus</i> spp	6.9	8.3	6.9	12.5	13.9	20.8	15.3	19.4	27.8
<i>Cladosporium</i> spp	2.8	6.9	4.2	6.9	6.9	13.9	9.7	11.1	19.4
<i>Exophiala</i> spp	1.4	0.0	0.0	0.0	1.4	0.0	1.4	0.0	1.4
<i>Fusarium</i> spp	1.4	0.0	0.0	2.8	1.4	2.8	1.4	2.8	2.8
<i>Nocardia</i> spp	8.3	13.9	1.4	6.9	9.7	20.8	22.2	8.3	25.0
<i>Paecilomyces</i> spp	1.4	1.4	1.4	0.0	2.8	1.4	2.8	1.4	4.2
<i>Penicillium</i> spp	1.4	4.2	2.8	1.4	4.2	5.6	5.6	4.2	9.7
<i>Sepedonium</i> spp	0.0	1.4	0.0	0.0	0.0	1.4	1.4	0.0	1.4
<i>Streptomyces</i> spp	1.4	4.2	1.4	2.8	2.8	6.9	5.6	4.2	9.7
<i>Trichosporon</i> spp	0.0	1.4	0.0	0.0	0.0	1.4	1.4	0.0	1.4
<i>Tritirhachium</i> spp	0.0	0.0	0.0	1.4	0.0	1.4	0.0	1.4	1.4
<i>Verticillium</i> spp	1.4	0.0	0.0	0.0	1.4	0.0	1.4	0.0	1.4
Unidentified	5.6	4.2	1.4	4.2	6.9	11.1	13.9	4.2	18.1

UCS : Upper conjunctival sac, LCS : Lower conjunctival sac, TUCS : Total % of fungus isolated in the upper conjunctival sac, TLCS : Total % fungus isolated in the lower conjunctival sac, TWS : Total % of fungus isolated on the wet swab technique, TDS : Total % of fungus isolated on the dry swab technique, TE : Total % of fungus isolated in the eye.

Table 2. Fungal isolates from the conjunctival sacs of herd 1 ponies(40 eyes)

(% of eyes sampled)

Fungus	Wet Swab		Dry Swab		TUCS	TLCS	TWS	TDS	TE
	UCS	LCS	UCS	LCS					
<i>Acremonium</i> spp	2.5	2.5	0.0	2.5	2.5	5.0	5.0	2.5	7.5
<i>Aspergillus</i> spp	0.0	0.0	0.0	2.5	0.0	2.5	0.0	2.5	2.5
<i>Cladosporium</i> spp	5.0	7.5	7.5	10.0	12.5	17.5	12.5	17.5	27.5
<i>Exophiala</i> spp	2.5	0.0	0.0	0.0	2.5	0.0	2.5	0.0	2.5
<i>Fusarium</i> spp	2.5	0.0	0.0	2.5	2.5	2.5	2.5	2.5	2.5
<i>Nocardia</i> spp	12.5	20.0	2.5	10.0	15.0	30.0	32.5	12.5	35.0
<i>Paecilomyces</i> spp	0.0	2.5	2.5	0.0	2.5	2.5	2.5	2.5	5.0
<i>Penicillium</i> spp	0.0	7.5	2.5	2.5	2.5	10.0	7.5	5.0	12.5
<i>Sepedonium</i> spp	0.0	2.5	0.0	0.0	0.0	2.5	2.5	0.0	2.5
<i>Streptomyces</i> spp	2.5	5.0	2.5	2.5	5.0	7.5	7.5	5.0	12.5
<i>Trichosporon</i> spp	0.0	2.5	0.0	0.0	0.0	2.5	2.5	0.0	2.5
<i>Verticillium</i> spp	2.5	0.0	0.0	0.0	2.5	0.0	2.5	0.0	2.5
Unidentified	5.0	7.5	0.0	5.0	5.0	12.5	12.5	5.0	17.5

UCS : Upper conjunctival sac, LCS : Lower conjunctival sac, TUCS : Total % of fungus isolated in the upper conjunctival sac, TLCS : Total % fungus isolated in the lower conjunctival sac, TWS : Total % of fungus isolated on the wet swab technique, TDS : Total % of fungus isolated on the dry swab technique, TE : Total % of fungus isolated in the eye.

Table 3. Fungal isolates from the conjunctival sacs of herd 2 ponies(32 eyes)

(% of eyes sampled)

Fungus	Wet Swab		Dry Swab		TUCS	TLCS	TWS	TDS	TE
	UCS	LCS	UCS	LCS					
<i>Acremonium</i> spp	0.0	0.0	3.1	0.0	3.1	0.0	0.0	3.1	3.1
<i>Aspergillus</i> spp	15.6	18.8	15.6	25.0	31.3	43.8	34.3	40.6	59.4
<i>Cladosporium</i> spp	0.0	6.3	0.0	3.1	0.0	9.4	6.3	3.1	9.4
<i>Fusarium</i> spp	0.0	0.0	0.0	3.1	0.0	3.1	0.0	3.1	3.1
<i>Nocardia</i> spp	3.1	6.3	0.0	3.1	3.1	9.4	9.4	3.1	12.5
<i>Paecilomyces</i> spp	3.1	0.0	0.0	0.0	3.1	0.0	3.1	0.0	3.1
<i>Penicillium</i> spp	3.1	0.0	3.1	0.0	6.3	0.0	3.1	3.1	6.3
<i>Streptomyces</i> spp	0.0	3.1	0.0	3.1	0.0	6.3	3.1	3.1	6.3
<i>Trüirhachium</i> spp	0.0	0.0	0.0	3.1	0.0	3.1	0.0	3.1	3.1
Unidentified	6.3	9.4	3.1	0.0	9.4	9.4	15.6	3.1	18.8

UCS : Upper conjunctival sac, LCS : Lower conjunctival sac, TUCS : Total % of fungus isolated in the upper conjunctival sac, TLCS : Total % fungus isolated in the lower conjunctival sac, TWS : Total % of fungus isolated on the wet swab technique, TDS : Total % of fungus isolated on the dry swab technique, TE : Total % of fungus isolated in the eye.

(Table 2,3).

Significantly more fungi were isolated from the lower conjunctival sac than from the upper conjunctival sac of ponies

in herd 1, whereas there was no statistical difference between the number of isolates from the upper and lower conjunctival sacs animals in herd 2. Collectively, fungi were

isolated from the lower conjunctival sac significantly more frequently than from the upper conjunctival sac ($p < 0.05$).

In both herds, there was no statistical difference between the wet swab and the dry swab culture technique.

Discussion

In our study, the most predominant species of fungus isolated in the two herds was *Aspergillus* spp (27.8%). This result was in agreement with other reports^{5,11,15,17}. Of the 399 fungal isolates recovered from 212 eyes by Eastwood¹⁵, 36.6% were *Aspergillus* spp which was the predominant fungus isolated. Lundvall¹¹, in a study of the normal equine ocular mycotic flora, isolated *Aspergillus* spp from 24% of 250 specimens as the most predominant isolate. Riis⁵ and Samuelson *et al*¹⁷ also recovered *Aspergillus* spp from 36% and 56% respectively of normal horses. *Aspergillus* spp have also been recovered in many instances from clinical cases of equine keratomycosis^{1,3,14,18-20,23}. This suggests that the fungi which exist in the normal eye may cause external eye disease in an opportunistic manner.

However, Moore *et al*²⁴ reported that *Cladosporium* spp and *Alternaria* spp were the most prevalent isolates in their study, and they only cultured *Aspergillus* spp from 2 of 40 normal horses. In one survey, *Alternaria* was the most common fungus isolated in 11 horses with keratomycosis⁴. *Cladosporium* spp was also one of the common fungi isolated in this study, but *Alternaria* spp was not recovered at all. Some studies isolated *Fusarium* spp in normal equine conjunctival sacs^{11,15}. *Fusarium* spp accounted for 8% and 18.1% of the isolates in the studies of Lundvall¹¹ and Eastwood¹⁵, respectively. *Fusarium* species are a common cause of human keratomycosis in southern Florida²⁵. Mitchell and Attleberger⁸ reported *Fusarium* keratomycosis in a horse at Auburn University. In our study, *Fusarium* spp accounted for only 2.4% of the isolates.

It was likely that the differences among studies were related to environmental factors and the number of sample taken. Although different fungal distributions between herd 1 and herd 2 were observed, total number of isolates were similar to other studies.

Nocardia spp and *Streptomyces* spp are not considered to be 'true' fungi, they are Gram-positive rods and filaments which sometimes show branching²⁶, hence many studies did not show these species in their results. If these fungi were discounted, the total incidence of fungal isolates in this study would be markedly decreased.

The distribution of isolates from the conjunctival sacs of ponies showed marked differences between the two herds in this study. Species of *Nocardia* and *Cladosporium* were predominant in herd 1, whereas *Aspergillus* was the predominant isolate in herd 2. These differences may be related to the immediate environment and management of the ponies. Moore *et al*²⁴ compared the prevalence of ocular microorganisms, including fungi between stabled horses and hospitalized horses in a more hygienic environment to investigate the effects of the horses' immediate surroundings on the prevalence of external ocular microbes. They found that the frequency of isolation of fungi was higher ($p < 0.01$) in stabled horses and suggested that these findings were related to environmental factors such as straw bedding, dirt floors and dust from the surroundings. This was also in agreement with the observations of Riis⁵ that the external ocular fungi in the horse may be transitory and related to random seeding from the environment. Surveys of the levels of fungal contamination of hay fed to horses in the UK have shown that between 60 and 80 percent of hay has a significant level contamination²⁷. In our study, management factors (diet, husbandry) and the degree of fungal contamination of the pasture would be more influential than any other factors, since this study was conducted in two herds which were subjected to the same climate during the same season in the same geographical location.

Fungi were cultured much more frequently from the lower conjunctival sac than the upper conjunctival sac. This result supports the literature which recommends the lower conjunctival sac as the most appropriate culture site. Under the action of gravity, the tears which will contain normal fungi of the external eye accumulate in the lower conjunctival sac. Therefore, the fungi are more likely to exist in the lower conjunctival sac. However, the fungi isolated from the lower conjunctival sac were not always the same as

those isolated from the upper conjunctival sac ; and in some eyes fungi were only isolated from the upper conjunctival sac and were not found in the lower conjunctival sac. This demonstrates that fungal culture of swabs obtained from both conjunctival sacs is a more reliable means of isolating all the fungal species present.

As yet no specific protocol has been established for culturing conjunctival microorganisms. However, wet swab culture techniques are generally recommended^{1-3,9,10,14,16,18} Hacker *et al*²² in a bacteriological study reported the wet culture technique resulted in bacterial growth in 88% of the cultured specimens, while the dry method resulted in growth in 72% of the cultured specimens. In our study, however, there was no significant difference between the wet swab and the dry swab methods. This difference may be attributed to the different grow characteristics of bacteria and fungi as well as sample size and technique used. In our study, some bacteria seemed to grow well in the wet swab

method, even though Sabouraud's dextrose agar was used.

Conclusions

The prevalence of fungal flora in the conjunctival sacs of normal pony herds in Hertfordshire, UK was investigated in 72 eyes(36 ponies). Fungi were isolated from 76.4% of the eyes. These isolate included 13 different genera. The most common fungal isolates were *Aspergillus* spp(27.8%), *Nocardia* spp(25.0%), *Cladosporium* spp(19.4%), *Penicillium* spp(9.7%) and *Streptomyces* spp(9.7%). *Nocardia* spp and *Cladosporium* spp were the predominant isolates in herd 1, whereas *Aspergillus* spp was the predominant isolate in herd 2. Collectively, there were significantly more fungi isolated from the lower conjunctival sac than from the upper conjunctival sac. There was no statistical difference between the wet swab and the dry swab culture technique.

Legend for figures

Fig 1. The most common fungi isolated in this study.

A : *Aspergillus* spp, 400X,

B : *Nocardia* spp, 312.5X,

C : *Cladosporium* spp, 400X,

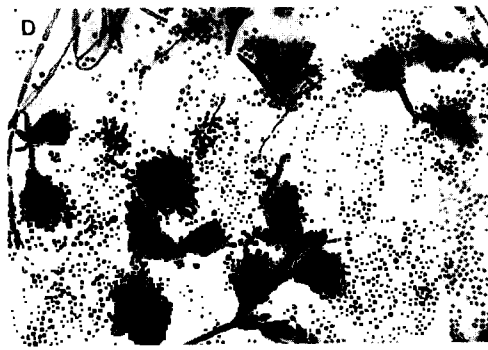
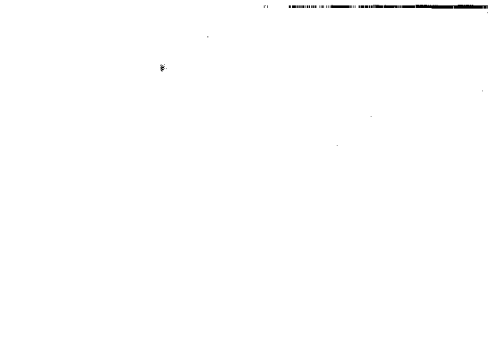
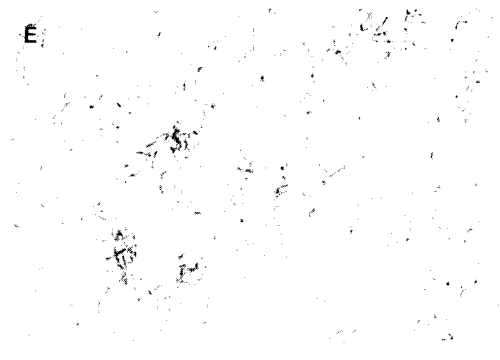
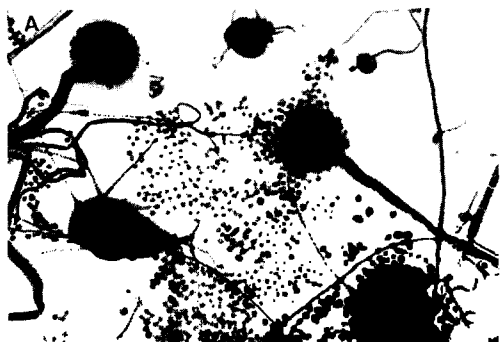
D : *Penicillium* spp, 400X,

E : *Streptomyces* spp, 312.5X,

F : *Acremonium* spp, 312.5X,

G : *Paecilomyces* spp, 312.5X,

H : *Fusarium* spp, 312.5X.



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